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UNIVERSITY OF ALBERTA

Comparison of host responses to *Giardia muris* in male and female mice

BY

©

Craig Wilfred Daniels

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science.

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

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Craig Daniels

9710-90 Ave., Fort Saskatchewan, AB

T8L-1M6

Oct. 8, 1993

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Comparison of host responses to *Giardia muris* in male and female mice submitted by Craig Wilfred Daniels in partial fulfillment of the requirements for the degree of Master of Science.

Dr. Miodrag Belosevic
Supervisor

Dr. John Holmes
Committee member

Dr. Glenn Armstrong
Committee member

Dr. Reuben Kaufman
Chairman

Oct. 6, 1993

ABSTRACT

I examined host responses of male and female C57BL/6 mice during primary and challenge infections with *Giardia muris*. There was a distinct difference in the course of infection between males and females. Male mice were shedding cysts in their feces both earlier and later after infection than females. Males were shedding cysts for at least 60 days after infection whereas females stopped by approximately day 20. While no differences were observed in the cyst output of the mice during the peak of infection, males had significantly more trophozoites in their intestines than females. The ability to resist a secondary infection was greater in females.

An examination of the serum antibody responses in the mice revealed a strong IgM response in both males and females early in the infection. However, striking differences between males and females in their IgG response were observed. Females had higher levels of IgG_{2b} and IgG_3 than males. Immunoblot analysis of parasite antigens indicated a strong response of both sexes to a group of approximately 30 kDa proteins and to 5 other higher weight proteins. However, no antigens were exclusively recognized by females.

Inflammatory responses during infection were also examined. No differences were observed in the ability of mice to recruit cells toward an inflammatory stimulus. However, in females the phagocytic ability of macrophages and the serum levels of tumour necrosis factor-alpha were higher than in males. Interferon-gamma was undetectable in the serum using the techniques employed.

The levels of intestinal disaccharidases were measured as an indicator of pathophysiology and of inflammation in the small intestine. Both males and females had decreases in enzyme activity during the time of highest parasite density in the intestine. However, only males had enzyme deficiency 20 days after infection. After challenge infections, however, females had significantly lower levels of two of the four disaccharidases measured.

Thus, a distinct difference exists in host responses of male and female mice to *G. muris*. These differences are discussed in relation to gender and T cell responses in infection and the current knowledge of the immunobiology of *G. muris* infections.

Acknowledgments

I would like to thank the members of my supervisory committee, Drs. Belosevic, Holmes and Armstrong for their support and guidance throughout this thesis. Also thanks to Dr. Anwar for his help as a member of my supervisory committee during the early stages of this thesis and to Dr. Kaufman for chairing my exam. All the members of my committee helped out with their comments on parts of this thesis and for their invaluable suggestions in meetings. Of course, as my supervisor, Dr. Belosevic contributed a great deal of time and energy to see this thesis through to the end.

Funding for this research was provided by NSERC and AHFMR Grants to Dr. M. Belosevic. I was also supported in part by teaching assistantships to myself from the Department of Zoology, University of Alberta.

The research was assisted by several people throughout this study and I would like to thank Feng Wang, Qiong Shen and Rebecca Guy for their assistance in the lab. Al Shostak contributed his help with statistics and with reviewing parts of this thesis. Numerous members of the Parasitology group contributed by discussing my work but I would especially like to thank Rebecca Guy and John Sovell for their discussions and encouragement.

On a personal note, much thanks to John, Rebecca and Jennifer for helping me through this degree and for putting up with me over the last several years.

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Chapter One: General introduction.

Giardia spp. are protozoan parasites of vertebrates and have a cosmopolitan distribution. The genus may have arisen from the earliest eukaryote lineage (Kabnick and Peattie, 1991) and *G. lamblia* has been a parasite of humans for greater than 2000 years (Faulkner *et al.*, 1989). *Giardia* has a simple asexual life-cycle with motile trophozoites living in the small intestine of the host and resistant cysts that are excreted into the environment. These cysts are ingested by the next host, excyst to produce two trophozoites, and begin a new infection (Figure 1.1).

Three species of *Giardia*, based upon morphological characteristics, are recognized by most authors: *G. lamblia* (=*intestinalis*), parasite of humans and several other mammals; *G. muris*, parasite of many rodents and birds; and *G. agilis*, parasite of tadpoles and frogs (Filice, 1952; Bertram *et al.*, 1984). Two additional, morphologically distinct, species of *Giardia* have been described from birds: *G. psittaci* from parakeets (Erlandsen and Bemrick, 1987) and *G. ardeae* from great blue herons (Erlandsen *et al.*, 1990).

G. lamblia infections in humans can cause diarrhea, abdominal cramping and malabsorption of nutrients. As a result, *G. lamblia* has received considerable attention for its high prevalence of infection. In the United States, *G. lamblia* is the most frequently reported cause of waterborne disease (Horwitz *et al.*, 1976). In Canada, it is the third most common cause overall. It is also the most commonly reported enteric infection in the Western Provinces and the Territories (Canada Communicable Disease Report, June 26, 1992). In developing countries reported prevalences range between five and 43% (Islam, 1990). Person-to-person transmission is common in children, with prevalences in day-care centres in Ontario close to 10% (Woo and Paterson, 1986) and in one day-care centre near Atlanta, Georgia as high as 54% (Black *et al.*, 1977). Male homosexuals are another group with high infection rates (Schmerin *et al.*, 1978; Kean *et al.*, 1979). Transmission via food-borne cysts has also been reported (Osterholm *et al.*, 1981; Peterson *et al.*, 1988).

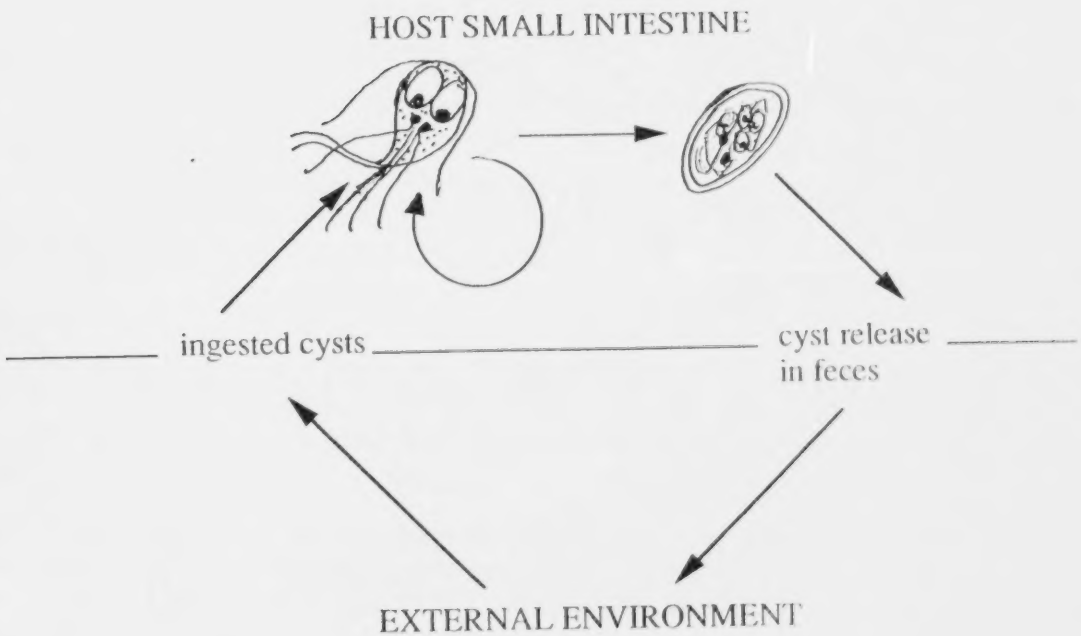

Figure 1-1. Life cycle of *Giardia muris*.

Control of Infection

The ability to control *Giardia* infections is associated with the ability of the host to mount an adequate immune response. For example, immunodeficient persons, such as patients with hypogammaglobulinemia, generally have severe symptomatic giardiasis and are unable to eliminate the parasite (Ament and Rubin, 1972). Patients with acquired immunodeficiency syndrome (AIDS) do not produce anti-*Giardia* antibodies (Janoff *et al.*, 1988); However, infections with *Giardia* and other enteric pathogens are not always associated with infection with the human immunodeficiency virus (Gazzard, 1990; Lucas, 1990). Epidemiological evidence indicates that persons living in *Giardia* endemic areas are less susceptible to infection and do not have symptomatic giardiasis. Visitors to these endemic areas are, however, invariably susceptible to infection and generally have symptomatic giardiasis (Istre *et al.*, 1984). This epidemiological evidence is supported by results from studies using animal models, which clearly show that prior exposure to *Giardia* results in long-lasting resistance to re-infection (Roberts-Thomson and Mitchell, 1978; Belosevic *et al.*, 1983). The original observation that T cell deficient nude mice have prolonged *G. muris* infections (Boorman *et al.*, 1973) and several studies on the role of antibodies in the control of *G. muris* in mice (Snider *et al.*, 1985; Butscher and Faubert, 1988; and reviews by denHollander *et al.*, 1988, and Heyworth, 1992) indicate the importance of the immune response, especially antibodies, in control of giardiasis.

Although the host immune response against *Giardia* is involved in the elimination of the parasite from the small intestine, the precise mechanism(s) of this process is not known. For example, we do not know why some strains of mice are more susceptible to infection than others. Differences in the ability of mice to mount appropriate immune responses are important in the elimination of *Giardia*, but exact mechanisms are unknown (see Chapter 2 for a review of immune responses to *Giardia*). The genetic background influences the susceptibility of the host to *Giardia* and is under non H-2, multigenic control (Belosevic *et al.*, 1984). The murine H-2 chromosome region is analogous to the human major histocompatability complex which codes for self recognition and many immune function genes. Roberts-Thomson *et al.* (1980) also observed that

several genes appear to influence susceptibility of mice to *G. muris* and that prolonged *G. lamblia* infections in humans may have a possible genetic basis. This evidence, based on blood group and HLA types in infected individuals, is uncertain and has been contradicted (Pettoello Mantovani *et al.*, 1990).

Sexual Differences in Infection

Gender specificity in the response and in the severity and prevalence of infection has been noted in many parasitic infections. There is often an immunological basis for this difference in susceptibility (see reviews by Alexander and Stimson, 1988; Brabin and Brabin, 1992). Males are generally more susceptible to parasites, but this is not always the case. For example, female mice are more susceptible to the larval stage of the tapeworm *Taenia crassiceps* (Sciutto *et al.*, 1991; Huerta *et al.*, 1992) and are more permissive hosts, and suffer greater mortality, when infected with *Schistosoma mansoni* (Eloi-Santos *et al.*, 1992). Male B10.129 (10M) ScSn mice are more resistant to cutaneous leishmaniasis than females, even though their antibody titres six months after infection are 1:16 compared to 1:64,000 in the females (Giannini, 1986). However, the males show a better cell-mediated response to the parasite and cell-mediated immunity is the controlling factor in leishmaniasis. In general, testosterone tends to have a suppressive effect on both cellular and humoral immune responses, whereas oestrogen enhances humoral responses but diminishes cell-mediated immunity (Alexander and Stimson, 1988).

A difference in susceptibility between male and female mice infected with *G. muris* has been noted (Roberts-Thomson and Mitchell, 1978; Faubert *et al.*, 1985) with infections in males lasting longer, and with higher cyst output in feces, than those in females. However, Heyworth (1988) concluded, based on cyst outputs during the acute phase of infection with *G. muris* infected BALB/c mice, that no difference in the infection exists between male and female mice. A possible difference in susceptibility to *G. lamblia* has been noted between males and females in human infections (Jokipii, 1971).

Appropriate inflammatory responses by the host are associated with the ability of mice to eliminate *G. muris* (Erlich *et al.*, 1983; Belosevic and Faubert, 1986) and testosterone has an anti-inflammatory effect

(Mooradian *et al.*, 1987). Sexual differences, specifically in testosterone levels, influence the inflammatory response of C57BL/6 mice infected with the nematode parasite *Brugia pahangi*, such that castrated males have both a greater inflammatory response and an increased ability to control the parasite (Nakanishi *et al.*, 1989). Thus, differences in infection with *G. muris* in male and female mice are likely to occur as a result of differential immune/ inflammatory abilities between the sexes.

The Thesis

The work described in this thesis was designed to examine differences in the infection with G. *muris* in male and female inbred C57BL/6 mice and to try to correlate differences in the host response with the course of infection. The experimental work had four components: 1) an examination of the course of infection; 2) an examination of antibody responses; 3) documentation of inflammatory responses and 4) an examination of intestinal pathophysiology. Components 2 and 3 were chosen because of the higher probability that antibody and inflammatory responses would be different between the sexes and because they are postulated to be important mechanisms in host defense to *Giardia* (denHollander *et al.*, 1988; Heyworth, 1992).

The second chapter is a review of the literature on host responses to *Giardia*, especially those examining responses of mice to *G. muris*. This review will allow the results of sex differences in infection to be placed in the broader context of what is known about the overall host response to this parasite.

In chapter three, I characterized the course of infection with *G. muris* in male and female C57BL/6 mice. The infections were monitored by determining cyst release in the feces and by enumerating numbers of trophozoites in the small intestine. Measurements were done both during the primary infection and after a challenge infection. I focused on determining the patterns of the infection, whether real differences existed between male and female mice of this strain, and when these differences were apparent.

In Chapter four, I examined the specific anti-parasite antibody responses in the mice. Measurements of whole serum IgG, IgM, IgA and the subclasses of IgG were measured during the primary and after

secondary infections. The specific IgG responses to parasite antigens were examined using Western blotting to determine whether males and females are recognizing different parasite antigens.

In the fifth chapter, I examined the inflammatory responses of mice to assess whether differences in inflammation can be correlated with the ability of the host to eliminate the parasite. Levels of the inflammatory mediators tumour necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ) were determined during primary and after challenge infections. Inflammatory cell function was examined by stimulating an inflammatory response in the peritoneal cavity. The recruitment and composition of the inflammatory cells, and phagocytosis by macrophages, were examined during primary and challenge infections.

In the sixth chapter, I examined pathophysiological changes in the small intestines of the mice as an indicator of intestinal disruption and indirectly of inflammation. Pathophysiology was assessed by measuring changes in intestinal disaccharidase activity (lactase, maltase, sucrase and trehalase).

The final section of this thesis is a synthesis of the results from the above chapters. I also address the basis for sexual differences in infections and how my results fit into what is known about gender differences and T cell control of infections.

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Chapter Two: Review of host responses to *Giardia*.

A large body of evidence has accumulated regarding host responses to *Giardia* infections. This work has highlighted that, while we know immune responses are important in parasite elimination and control, we still do not know the exact mechanism involved. Differences in innate or induced immune responses in a variety of inbred mouse strains have given some answers, but there are no data on differential immune responses between the sexes. This review will give an overview of the current knowledge of immune responses against *Giardia*. One important point to keep in mind when reviewing this literature is that all the work done, with only one or two exceptions, has examined immune responses in female mice but not males. The study of Erlich *et al.* (1983) compared antibody responses of male susceptible mice with those of females from a resistant strain, but did not compare males and females of the same strain of mice.

I. Immune Responses.

A. Humoral Immunity: The humoral components of the immune system are thought to play a major role in the elimination of *Giardia* from the host. Mice deficient in antibody after treatment from birth with anti-IgM rabbit serum (and thus deficient in all antibody classes) have chronic infections with *G. muris* (Snider *et al.*, 1985). The anti-IgM antibody treatment used above is directed against the μ (heavy) chain of IgM on the surface of B cells. Surface IgM is found on all immature and developing B cells (Roitt *et al.*, 1985).This, therefore, will eliminate all or most B cells in the treated mice. B cell deficient mice expressing the *xid* gene are also more susceptible (Snider, Skea & Underdown, 1988). In *G. lamblia* infections in humans, hypogammaglobulinemic patients have increased prevalence and severity of infection (Ament and Rubin, 1972).

In vitro studies

Complement is that part of the humoral immune system that refers to a complex series of proteins involved in host defense (Roitt *et al.*, 1988). Complement has several functions involved with the destruction of foreign organisms. It opsonizes (coats) organisms to enhance the phagocytic ability of macrophages and neutrophils and also enhances the

ability of antibody coated particles to be phagocytosed. It can also attract phagocytic cells and can kill targets directly by forming a membrane attack complex that forms holes through the targets cell membrane. Formation of this membrane complex is initiated by bound antibody or directly initiated by complement. Trophozoites of *G. lamblia* (Hill *et al.*, 1984; Deguchi *et al.*, 1987) and *G. muris* (Belosevic and Faubert, 1987; Butscher and Faubert, 1988) are highly susceptible to antibody-mediated complement lysis *in vitro*. Specific anti-parasite IgM and IgG_3, but not IgA, monoclonal antibodies (MAb), are able to fix complement and kill *G. muris* trophozoites *in vitro* (Heyworth, 1992). However, IgA does not possess the ability to activate complement (Roitt *et al.*, 1985) so this is not surprising. Trophozoites of *G. lamblia* can activate complement both through the classical pathway (Hill *et al.*, 1984) and the alternative pathway (Deguchi *et al.*, 1987). Antibodies are also cytotoxic to *G. lamblia* in the absence of complement (Nash and Aggarwal, 1986).

Anti-*Giardia* antibodies are effective opsonins that facilitate adherence and phagocytosis (= killing) of trophozoites by neutrophils and macrophages (Kaplan *et al.*, 1985). This antibody-dependent cellular cytotoxicity (ADCC), has been demonstrated by several authors (Smith *et al.*, 1983; Kanwar *et al.*, 1986; Belosevic and Faubert, 1986a; Hill and Pearson, 1987).

Monoclonal antibodies (IgM and IgG_3) produced against *G. lamblia* agglutinate trophozoites (Guy, 1989; Heyworth *et al.*, 1989) and decrease their attachment to plastic culture vessels (Guy, 1989). Attachment of trophozoites is necessary for maintaining trophozoites within the intestine. Therefore, interference with attachment may be effective in reducing trophozoite numbers within the host.

In vivo studies

Several authors have presented evidence for the presence of anti-parasite antibody within the intestines of infected mice. *G. muris* trophozoites isolated from resistant BALB/c mice have IgA and IgG, but not IgM, bound on their surface while trophozoites from susceptible nude mice (T cell deficient) have little or no bound antibody on their surface (Heyworth, 1986). BALB/c mice depleted of T helper cells also have less antibody bound on trophozoite surfaces (Heyworth, 1989). However, in

this study, Heyworth reported little bound IgG on trophozoite surfaces as opposed to his earlier study with mice of the same strain (Heyworth 1986). Underdown *et al.* (1981) and Erlich *et al.* (1983) also found IgA in the intestine but found that susceptible C3H/He mice had higher levels of IgA in their small intestine than the resistant BALB/c mice. However, the increased levels of IgA in the C3H/He mice were found later in the infection while the BALB/c mice had a peak IgA response at the time of parasite expulsion.

Snider and Underdown (1986) examined both intestinal and serum levels of IgM, IgG and IgA in two strains of mice (C57BL/6 and BALB/c) and found only IgA in the intestinal secretions of these mice. IgM was not detected in the serum or intestine and IgG was noted only in serum. The appearance of IgA in the intestine was associated with the beginning of parasite expulsion in both mouse strains. In *G. lamblia* infections in mice, the number of lamina propria cells positive for IgA declines during peak parasite loads but increases at the time of parasite decline (Vinayak *et al.*, 1991). In the same study, the authors found that IgM positive cells began to increase in number at the time of peak parasite numbers while numbers of IgG positive cells significantly increased at the time of parasite decline.

Humans often show higher antibody titres to *G. lamblia* antigens than unexposed controls, although the presence of cross reactive antigens confuses this information (Jokipii *et al.*, 1988). Other studies have shown no differences between individuals in serum IgA, IgG, IgM and IgE antibody titres (Jones and Brown, 1974). Female patients have initially higher antibody titres (measured against all serum immunoglobulins but primarily to IgG) than males, but these differences disappear later (> 60 days) in the infection (Jokipii *et al.*, 1988).

Butscher and Faubert (1988) showed that intra-peritoneal injection of infected mice with an anti-*G. muris* IgM MAb lessened parasite burden in the small intestine although they did not determine the mechanism involved. These MAb's were able to agglutinate trophozoites and to kill in the presence of complement. Therefore, the available evidence indicates that anti-parasite antibody, complement, and their interaction, may participate in host defense against *Giardia*.

B. Cell Mediated Immunity: Several classes of immune cells are thought to play a role in the elimination of *Giardia* infections. These include T and B lymphocytes, neutrophils and macrophages. Probably the best studied is the involvement of T cells in host defense against *Giardia*. The original observation that T cell deficient nude mice have prolonged *G. muris* infections (Stevens *et al.*, 1978) has led many authors to conclude that the elimination of *G. muris* trophozoites is a T cell-dependent phenomenon.

T cells:

Intraepithelial lymphocytes (primarily T cells) in mice infected with *G. muris*, while initially decreasing in number one week after infection, increase in number during the infection (Gillon *et al.*, 1982). The numbers of T lymphocytes in Peyer's patches increase in infected BALB/c mice, although infection does not lead to altered percentages of T cell subclasses. These subclasses include T helper cells (Th), T suppressor cells (Ts) and cytotoxic T cells (Tc) (Carlson *et al.*, 1986a). Nude mice have lower numbers of L3T4$^+$ (equals Th) cells in their Peyer's patches when compared to BALB/c mice (Heyworth *et al.*, 1985a). Selective depletion of various T cell populations shows that, while mice lacking Ts and Tc cells clear *G. muris* at a normal rate, mice lacking Th cells do not clear the infection (Heyworth *et al.*, 1987). Similarly, Carlson *et al.* (1986b) found that clearance of *G. muris* was associated with a Th to Ts ratio greater than five.

In *G. lamblia* infections of suckling mice, an influx of Lyt 2.2$^+$ (Ts/c) cells into the intraepithelial and lamina propria areas of the jejenum were observed during parasite establishment and during the time of peak parasite numbers. The number of L3T4$^+$ (Th) cells only increased during the time of the decline in parasite numbers 17-21 days after infection (Vinayak *et al.*, 1991).

A recent paper reported some success in partially immunizing mice against infections with *G. lamblia* using a 56 kDa molecule from *G. lamblia* (Vinayak *et al.*, 1992). The mice, by recruiting helper T cells faster than non-immunized mice, were able to eliminate the parasite sooner, and keep trophozoite numbers lower, than untreated mice. Therefore, helper T cells are necessary for parasite control and are

associated with parasite expulsion. Suppressor T cells increase in number early in the infection but are later replaced by an influx of Th cells.

The *in vitro* responses of T cells from *G. lamblia* infected mice were not different from uninfected controls when the cells were exposed to the mitogen phytohaemagglutinin (PHA) (Radhakrishna *et al.*, 1984). However, lymphocytes from Peyer's patches of *G. muris* infected BALB/c mice show a specific proliferative response to parasite antigen that peaks approximately 14 days after infection and remains elevated for greater than 60 days after infection (Hill, 1990).

Thus, T helper cells are a necessary component of an effective immune response against *Giardia*. However, they may play a supporting, rather than a direct, role as they are known to facilitate specific immune responses to parasite antigens. For example, mice depleted of Th cells have an impaired anti-*G. muris* IgA response as seen by the lack of bound antibody on the surface of trophozoites when compared to untreated mice (Heyworth, 1989).

Macrophages:

The original evidence for the role of macrophages in the host response to *Giardia* was provided by Owen *et al.* (1981) who showed, using electron microscopy, that Peyer's patch macrophages engulfed *G. muris* trophozoites. These macrophages were in close contact with intraepithelial lymphocytes and may present antigen to these lymphocytes (Owen *et al.*, 1981). Heyworth *et al.* (1985b) examined the immune cells in the intestinal lumen of uninfected and *G. muris* infected BALB/c mice and found that T cells and macrophages in the intestinal lumen were often observed in close contact with trophozoites. Macrophages isolated from human blood ingest and kill *G. lamblia* trophozoites and will produce an oxidative burst after ingestion of parasites (Hill and Pearson, 1987). Bone marrow derived macrophages exhibit spontaneous killing of *G. lamblia* trophozoites *in vitro* and this effect is greatly increased by the treatment of macrophages with interferon-gamma (IFNγ) and LPS (bacterial lipopolysaccharide) (Belosevic and Daniels, 1992).

Other immune cells:

While many of the large granular lymphocytes present in the mouse intestinal mucosa are lymphocytes expressing natural killer (NK) activity (Tagliabue *et al.*, 1982), mice deficient in NK cells do not show differences in the course of infection as compared to mice with normally active NK cells (Heyworth *et al.*, 1986).

In *G. muris* infected rats, the numbers of mast cells increased significantly over controls from four to eight weeks after infection (Kasprzak *et al.*, 1991); however, the authors in this study did not state the numbers of cells found, only that there was an increase.

As mentioned above, *in vitro* studies have shown that anti-*Giardia* antibodies are effective opsonins for neutrophils and macrophages (Kaplan *et al.*, 1985) and that antibody-dependent cellular cytotoxicity has been demonstrated by Smith *et al.* (1984), Kanwar *et al.* (1986) and Belosevic and Faubert (1986a). Thus, both cellular and humoral components of the immune system play a role in host defense against *Giardia*.

C. Immune suppression: Three studies have presented evidence that *G. muris* infection may cause some degree of immune suppression in the host. Brett (1983) reported that mice infected with *G. muris* were less able to mount immune responses to the thymus-dependent antigen sheep red blood cells (SRBC) but that no suppression could be demonstrated in the response to a thymus independent antigen (TNP-lipopolysaccharide). As well, adherent peritoneal exudate cells from infected mice were slightly less cytotoxic to tumour-cell targets than cells from uninfected mice.

Belosevic *et al.* (1985a) studied the antibody response of both spleen and mesenteric lymph node (MLN) cells to SRBC in *G. muris* infected susceptible and resistant mice. Infected mice of both strains were less responsive to the antigen, as measured by a plaque forming cell assay, than uninfected mice and the suppression occurred during the time of greatest parasite load in the small intestine. As well, transfer of MLN cells (but not of spleen cells) to uninfected mice caused a depression in the antibody response of recipient mice to SRBC. Intravenous administration of a trophozoite extract to uninfected mice also decreased their antibody responses to SRBC. Belosevic *et al.* (1985b) also examined these responses *in vitro* and found similar results. They also isolated the cells involved

with the suppressor activity and found that the plastic-adherent cells (probably macrophages) were involved.

The above studies suggest that the host is focusing its energy on the parasite and is suppressing or unable to mount responses to other immune stimuli.

D. Antigen recognition and antigenic variation: A number of antigens recognized by the host have been described from *Giardia* and range in size from 23.5 to 225 Kilodaltons (kDa) (reviewed by denHollander *et al.*, 1988). Some of these are stage specific and are found only on cysts (Reiner *et al.*, 1989). As well, a *G. lamblia* surface antigen with lectin activity, most specific to mannose-6-phosphate, has been described (Lev *et al.*, 1986). This antigen has been implicated *in vitro* to be important in attachment of trophozoites to host cells (Magne *et al.*, 1991). Clones of *G. lamblia* with different antigenic profiles differ in infectivity and virulence in mongolian gerbils (*Meriones unguiculatus*) (Udezulu *et al.*, 1992). Trophozoites grown *in vitro* also express different surface antigenic profiles when grown under different environmental conditions (Reiner and Gillin, 1992).

Trophozoites of *G. lamblia* undergo antigenic variation both *in vitro* and *in vivo* (Nash, 1992). Clones of trophozoites spontaneously change their antigenic makeup *in vitro* (Nash, 1992) and *in vivo* change their surface antigen makeup at approximately seven days after infection in mongolian gerbils (Aggarwal and Nash, 1988). These antigenic changes have been documented in experimental human infections with an antigenic change found around day 22 post-infection (Nash *et al.*, 1990). Infections with *G. lamblia* in both nude (T cell deficient but can produce antibody responses) and severe combined immunodeficient (scid) mice show that the antigenic change occurs only in the nude mice. While nude mice produce specific anti-parasite antibodies (although limited to T independent antigens) the scid mice do not suggesting that the antigenic change observed is driven by host antibody responses to the parasite (Gottstein and Nash, 1991).

If parallels can then be drawn to infections with *G. muris* in mice then a similar antigenic shift should occur. What effect this may have on

the course of infection is unclear. As well, the likely possibility that different strains of *G. muris* exist has not been investigated.

II. Inflammatory Reactions:

While the processes and cells involved in inflammation are part of the immune system they form a distinct set of reactions within this system. Inflammation was classically defined by four cardinal signs: rubor (redness), tumor (swelling), calor (heat) and dolor (pain). These are the result of an extremely complex series of reactions occurring in an animal after injury. Three major events occur in inflammation that are designed to increase the body's ability to deal with the injury. These events are: 1) increased blood supply to the injured area; 2) increased vascular permeability to cellular traffic through retraction of cells, particularly in the post-capillary venules, and 3) the attraction (chemotaxis) to the site of inflammation of scavenger cells, such as neutrophils and macrophages, which remove invaders and damaged tissue (Roitt *et al.*, 1985).

Several lines of evidence suggest that inflammatory reactions play a major role in infections of *G. muris* in mice and that inflammatory ability is correlated with host genotype. Recruitment of thioglycollate-elicited peritoneal inflammatory cells is greater in resistant B10.A mice than in susceptible A/J mice early in, and during the elimination phase of, infections with *G. muris*. However, mice of both strains recruit lower numbers of cells when infected (Belosevic and Faubert, 1986b). This is further evidence for inflammation occurring in the intestine during infection as ongoing inflammatory events in one location suppress inflammation in other sites (Castro *et al.*, 1980; Normann *et al.*, 1991).

Mast cell deficient W^f/W^f mice develop chronic *G. muris* infections (Erlich *et al.*, 1983) and treatment of resistant BALB/c mice with the anti-histamine/anti-serotonin drug cyproheptadine causes prolonged infections (Erlich *et al.*, 1983). W^f/W^f mice also have a deficient eosinophil response (Vadas, M.A., unpublished, quoted by Mitchell *et al.*, 1982) and a stem cell defect leading to a macrocytic anemia (Russell and Bernstein, 1966). These defects could also lead to an impaired inflammatory response. C3H/HeN mice, which are susceptible to *G. muris*, are non-responsive to the histamine sensitizing factor of *Bordetella pertussis* (Bergman and Munoz, 1968). Intestinal *Trichinella spiralis* infections in mice, which

cause a large intestinal inflammatory response, result in lower numbers of *G. muris* trophozoites in concurrent infections (Roberts-Thomson *et al.*, 1976). Thus, the available evidence suggests that inflammation during *G. muris* infection may play a critical role in the elimination of this parasite from the small intestine.

The identity of the cells involved in the inflammatory response during *Giardia* infections is unknown. Mast cells are a major source of inflammatory mediators upon antigen challenge. However, the major sensitizing trigger of mast cell release, IgE, is not elevated in human infections with *G. lamblia* (Jones and Brown, 1974; Geller *et al.*, 1978). Protozoan infections do not produce elevated IgE levels although mice immunized with *Toxoplasma gondii* will do so when treated with the IgE adjuvant aluminum hydroxide (Watanabe and Kobayashi, 1989). This is not to say that there is no anti-parasite IgE produced, but IgE has not been detected in protozoan infections (Scaglia *et al.*, 1979). Certain compounds can trigger mast cell release directly (Kaliner, 1987) but known *G. lamblia* antigens (denHollander *et al.*, 1988) do not fit this category of compounds.

Other cells, such as enterochromaffin cells, might produce compounds that could trigger mast cell degranulation directly and stimulate inflammatory mediator production. Alternatively, these cells may themselves be a major source of inflammatory mediators. Macrophages and granulocytes attracted to the site of infection also produce inflammatory mediators. The mechanism whereby *Giardia* antigens trigger inflammatory reactions is unknown. Clearly, the assessment of inflammatory mediators during primary and challenge infections would increase our understanding of the importance of inflammatory reactions in giardiasis.

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Chapter Three: The course of *Giardia muris* infection in male and female C57BL/6 mice.

INTRODUCTION

An animal model of giardiasis was first described by Roberts-Thomson *et al.* (1976) who examined the pattern of cyst release and numbers of trophozoites of *Giardia muris* in a primary infection in female outbred Swiss albino mice (CF-1). These mice had a maximum cyst output in their feces five to 14 days after infection with numbers averaging log four to log five cysts excreted per two hour collection and had cleared the infection in three to four weeks. A similar pattern was observed when they examined the numbers of trophozoites in the small intestine. The numbers of trophozoites recovered reached log six, from five to 14 days after infection. A correlation between cyst release in feces and trophozoite numbers in the intestine was observed. Mice given 10,000 cysts excreted higher numbers of cysts and had higher numbers of trophozoites in their small intestine, during the acute phase of the infection, than animals given 100 or 1000 cysts. However, these animals also cleared the infection faster than the animals given lower numbers of cysts. The acute phase of infection is the time in the infection where cyst release and trophozoite numbers are at their highest, usually at days six through 12 after infection.

Owen *et al.* (1979) and Belosevic and Faubert (1983a) repeated much of this work and found a similar, reproducible, pattern of infection in mice and also concluded that cyst excretion in feces correlates with numbers of trophozoites. However, Olveda *et al.* (1982) found that while cyst output in feces correlates with trophozoite numbers in the peak period of infection, at other times trophozoites are present when no cysts are being passed in feces. Several other investigators have described the varying course of infection of *G. muris* in different mouse strains. Whereas certain inbred strains have the ability to eliminate the infection in three to four weeks, others show a characteristic inability to eliminate the parasite from their small intestine (Roberts-Thomson and Mitchell, 1978; Brett and Cox, 1982; Belosevic *et al.*, 1984). Mice also show an acquired resistance to reinfection after primary infections and are reinfected only transiently (Belosevic and Faubert, 1983b; Underdown *et al.*, 1981).

A difference in susceptibility has also been reported between male and female mice. Faubert *et al.* (1985) examined cyst release in three strains of mice (CD-1, A/J and B10.A) and observed that males, while having similar acute phase infections as females, shed cysts in their feces longer than females. The female mice had no trophozoites in their intestines on day 80 after infection while 40% of males were still positive. However, Heyworth (1988) examined cyst release in male and female BALB/c mice and concluded that no difference exists between the sexes in the time-course of the infection. Heyworth did not examine trophozoite numbers in the intestines of the animals in his study. A possible sexual difference in susceptibility to *G. lamblia* has also been noted in human infections (Jokipii, 1971).

The pattern of infection in C57BL/6 mice, as measured by cyst release in feces, has been examined in females (Belosevic *et al.*, 1984, Snider and Underdown, 1986) but not males, and the course of infection after a challenge infection has not been examined in these mice. Trophozoite numbers in the C57BL/6 mice have been examined in female but not male mice by Snider and Underdown (1986). To characterize the course of infection in male and female C57BL/6 mice and to further characterize sexual differences in murine giardiasis I examined the pattern of cyst release in primary and challenge infections and enumerated trophozoite loads in the small intestine of the mice to be used in my studies.

MATERIALS AND METHODS

Mice

Specific pathogen free, five to seven week old, inbred male and female C57BL/6 mice were used in all experiments. Mice were purchased from Charles River Breeding Laboratories (St. Constant, Quebec) or Taconic Laboratories (Germantown, N.Y.) and maintained in the University of Alberta Biosciences Animal Services Facility. Mice were housed three to five per cage with 0.22 μm filter top cages and autoclaved bedding. Food and water were provided *ad libitum*.

Parasite

The *G. muris* used in the experiments was initially isolated from a Golden hamster by Roberts-Thomson *et al.* (1976). The parasite was maintained in the laboratory by passages of 10,000 cysts/mouse administered by gavage to either C57BL/6 or CD-1/CRBr mice (Charles River Breeding Laboratories, St. Constant, Quebec). Passages were done every two weeks.

Isolation and enumeration of cysts

Cysts were isolated from the feces of mice using the sucrose gradient technique of Roberts-Thomson *et al.* (1976). Collected feces were emulsified in deionized water with a wooden applicator stick, layered onto a 1 M sucrose solution in 12 X 75 mm glass test tubes, and centrifuged for 15 minutes at 400 X *g*. The cysts trapped at the sugar-water interface were removed with a pasteur pipet. The cysts were washed in deionized water by centrifugation at 600 X *g* for 10 min, the supernatant discarded and the pellet re-suspended in 1 ml of deionized water. Cysts were counted using a haemocytometer.

Enumeration of trophozoites

The number of trophozoite within the small intestine was determined by dividing the small intestine into four equal pieces, slitting open the intestine and lightly scraping the mucosa in a known volume of phosphate buffered saline (PBS) in 15 X 75 mm tubes. Tubes were incubated at 37° C for two hours and were shaken approximately every 15 minutes. Tubes were then placed on ice, to prevent trophozoite attachment to tubes, and the numbers of trophozoites counted using a haemocytometer.

Experimental design:

Cyst output in feces

Male and female C57BL/6 mice were inoculated on day zero with 1000 cysts each of *G. muris* suspended in 0.2 ml of deionized water and administered to unanaesthetized mice by gavage. On collection days mice were individually placed in false bottom cages for two hours, between 9:30 and 11:30 a.m., and the feces collected and weighed. The cysts were

isolated and counted as described above and the cyst output for each mouse expressed as cysts per gram feces. Mice producing no feces on a particular collection day were not counted in that days' analysis. On day 70 the infection was eliminated by treating all mice for three consecutive days with 1 mg of metronidazole per day (Metronidazole Injection USP, 5 mg/ml, Abbott Laboratories Ltd., Montreal) administered by gavage. Mice were checked for cysts in their feces on two consecutive days after treatment and one mouse chosen at random, killed and the small intestine examined for trophozoites. After the mice had been allowed to rest for ten days they were reinfected with 1000 cysts and the cyst output measured every second day for ten days.

Comparable experiments of cyst release from male and female mice were done twice; once on a three day examination schedule (Experiment A) and once with examinations done every five days (Experiment B). Males had from eight to 10 mice per group for the three day examinations and from 10 to 14 per group for the five day examinations. Females had from 8 to 10 mice per group for the three day examinations and five to 10 per group for five day examinations. Cyst release was compared between males and females with a Mann-Whitney U test as the data were not normally distributed. Probability values of less than 5% were considered significant.

Challenge infections were done twice, once with five mice of each sex and once with 15 male and 10 female mice.

Latent period

The latent period, the first day after infection when at least 50% of the mice are passing cysts in their feces, was determined in male and female mice. The feces were examined daily on days two through five after infection in 11 male and 9 female mice.

Trophozoites

In separate experiments, mice were infected on day 0 with 1000 cysts and trophozoite counts were done every ten days. The numbers of trophozoites were determined in two separate experiments, experiment one examined trophozoite numbers on days 10, 20 and 30 after infection while experiment two enumerated trophozoites every 10 days from day 10

to day 50. Experiment one had seven to ten mice per group and experiment two had five mice per group. Trophozoite numbers after a challenge infection were determined ten days after a challenge with 1000 cysts in nine males and eight females.

RESULTS

Cyst Release in male and female mice

The cyst release data were graphically presented in three ways. Figure 3-1 presents the cyst output as abundance of infection, where abundance is defined as the total number of parasites found divided by the total number of hosts examined, including mice in which no parasites were observed. The data was also presented as mean intensity (Fig. 3-2), which is the mean number of parasites present divided by the number of positive hosts examined. Table 3-1 gives the sample sizes and the prevalence of infection, where this is expressed as a percentage of the number of positive animals present divided by the number examined. The terms are used as set out in Margolis *et al.* (1982).

The pattern of cyst release was similar in both trials. During the acute phase of the infection (approximately day six through 10) the mice were passing similar numbers of cysts in their feces. No cysts were ever observed in the feces of the female mice after day 18 in experiment A and day 20 in experiment B (Figs. 3-1 and 3-2). In contrast, many male mice continued to shed cysts in their feces for at least 60 days, the last examination point in this study. In experiment A, not all of the male mice were shedding cysts in the feces at any one time after day 15 (Table 3-1, Experiment A). In addition, some male mice were not shedding cysts on days six and nine after infection. This was an unexpected observation as this is the time of peak trophozoite numbers in infection and cysts should have been released in the feces. In the other experimental trial (B), even during the acute phase of the infection there were both males and females which were not passing cysts in their feces.

Cyst release as plotted by abundance (Fig. 3-1) did show a more variable pattern of cyst release than the intensity values (Fig. 3-2). The abundance data was heavily influenced by prevalence as the intensity values were nearly constant. Increases in the numbers of mice shedding

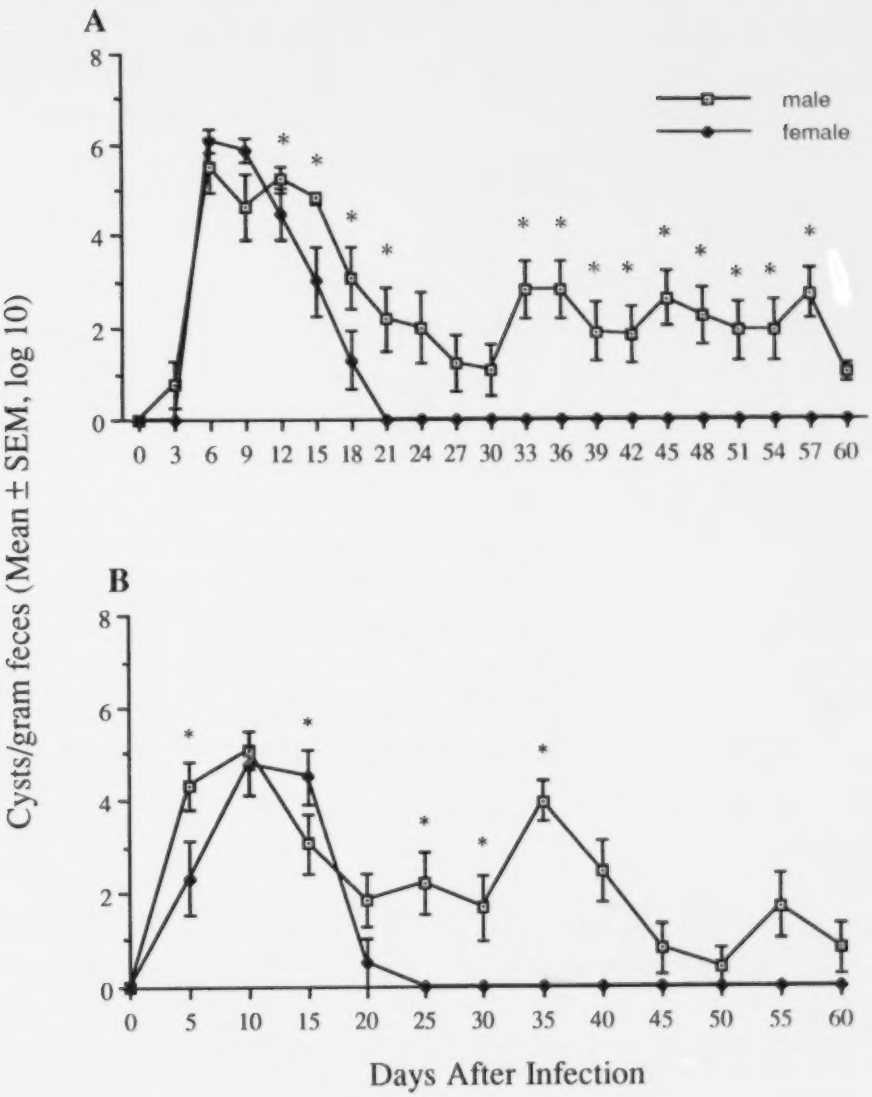

Figure 3-1. Abundance of *Giardia muris* infection in male and female C57BL/6 mice as measured by cyst release in feces. Abundance is defined as the mean number cysts released for all mice examined, including negatives. Results are the geometric mean ± SEM of cysts released per gram feces for: A) n= 8 to 10 mice of each sex and B) n= 10 to 14 male and 5 to 10 female mice. Asterisks represent significant differences between that group and the corresponding group of the opposite sex ($P<0.05$, Mann-Whitney U test).

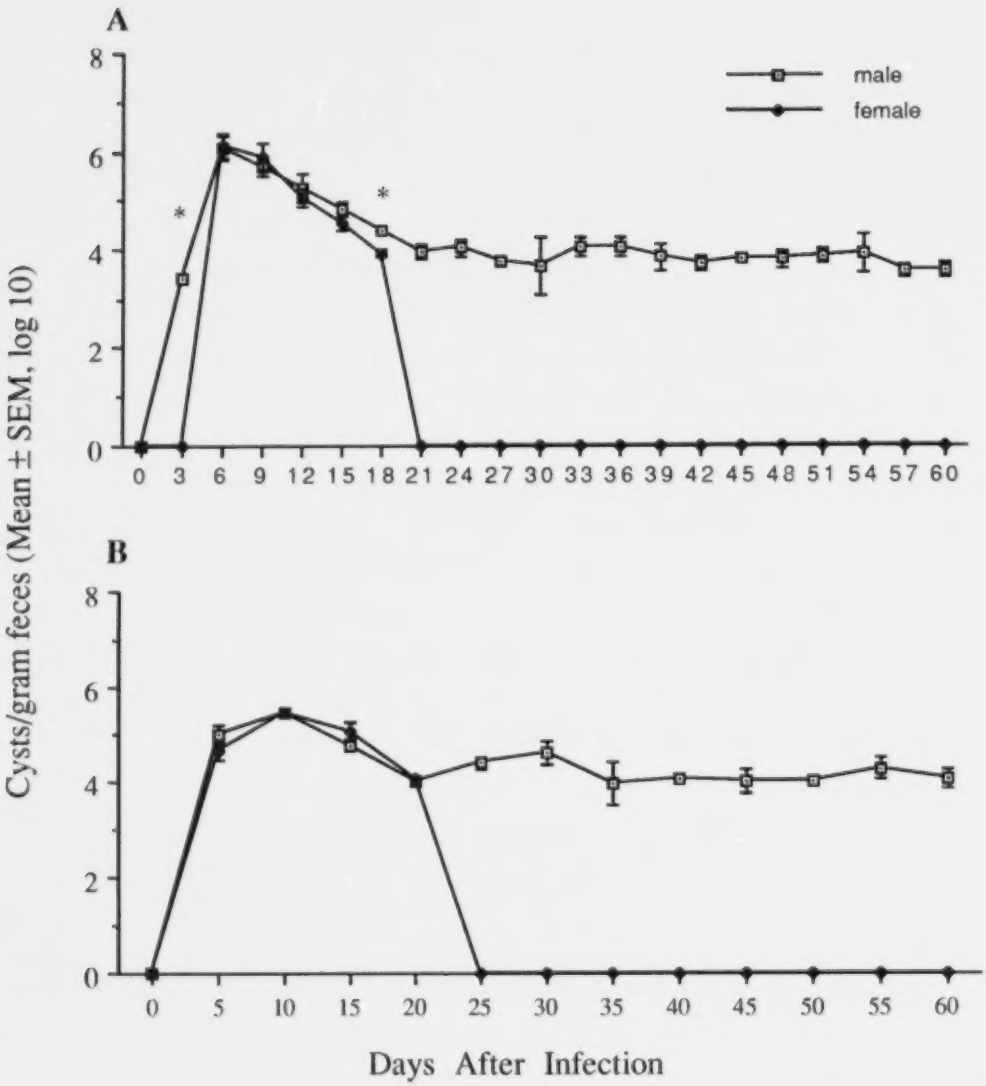

Figure 3-2. Intensity of *Giardia muris* infection in male and female C57BL/6 mice as measured by cyst release in feces. Intensity is defined as the mean number cysts released per positive animal. Results from two experiments are presented (A and B) and are the geometric mean number ± SEM of cysts released per gram feces. Asterisks represent significant differences between that group and the corresponding group of the opposite sex ($P<0.05$, Mann-Whitney U test). All values after d18 (A) and d20 (B) are significant.

Table 3-1. Prevalence of infection (#infected/#examined) and sample sizes for the measurement of number of cysts in the feces of male and female C57BL/6 mice during a primary *Giardia muris* infection.

Experiment A					Experiment B				
	Male		Female			Male		Female	
Days p.i.	# positive/ #examined	%	# positive/ #examined	%	Days p.i.	# positive/ #examined	%	# positive/ #examined	%
3	2/10	20	0/9	0	5	12/14	86	5/10	50
6	10/11	91	9/9	100	10	13/14	93	7/8	88
9	9/11	82	9/9	100	15	9/14	64	8/9	89
12	10/10	100	8/9	89	20	6/13	46	1/8	13
15	10/10	100	6/9	67	25	6/12	50	0/9	0
18	7/10	70	3/9	33	30	4/11	36	0/5	0
21	5/9	56	0/8	0	35	9/10	90	0/4	0
24	4/8	50	0/9	0	40	6/10	60	0/4	0
27	3/9	33	0/9	0	45	2/10	20	0/4	0
30	3/10	30	0/9	0	50	1/10	10	0/5	0
33	7/10	70	0/9	0	55	4/10	40	0/5	0
36	7/10	70	0/9	0	60	2/10	20	0/5	0
39	5/10	50	0/9	0					
42	5/10	50	0/9	0					
45	7/10	70	0/8	0					
48	6/10	60	0/8	0					
51	5/10	50	0/8	0					
54	5/10	50	0/9	0					
57	7/9	78	0/9	0					
60	2/8	25	0/8	0					

cysts changed the mean values such that the abundance plot mirrored the prevalence. For example, on day 35 after infection (Experiment B) the male mice showed approximately a two log increase in the mean number of cysts released (in abundance) as more of the mice examined were passing cysts in their feces (Fig. 3-1). While on day 30 5/12 male mice were passing cysts on day 35 13/14 mice had cysts present in their feces (Table 3-1). The number of cysts was down to a mean of log one and 6/14 mice were passing cysts on day 45.

The intensity data indicates that, while more males were passing cysts at this time period, the males shedding cysts were all producing virtually identical numbers of cysts in their feces. All of the males examined shed cysts in their feces at some point later in the infection. However, some did so only once after approximately day 20 of infection. The males, then, have the same pattern of cyst release during the acute phase of the infection as do females but release cysts sporadically after this point while females do not.

Latent period

Differences in the latent period of the mice were observed between the sexes with the male latent period being four days (with 6/9 mice passing cysts) and the latent period in females being five days with 9/9 mice passing cysts (Table 3-2). Female mice were first positive on day four (2/9) while the males were first positive on day three (3/9).

Cyst release after challenge infections

Data from the two experiments were combined. Cyst release after a challenge infection was different between the sexes with female mice having observable cysts in their feces only on day two after challenge with two of 15 females shedding cysts (Table 3-3). Mean numbers of cysts produced was higher in the males and sustained over the ten days. However, not all of the males were passing cysts at any one time and some males (8/20) never shed cysts in their feces.

Trophozoite numbers in the small intestine

Data from two experiments during the primary infection were combined for analysis. Examination of the data showed high variance to

Table 3-2: The Latent period of male and female C57BL/6 mice during a primary infection with *Giardia muris*. Values are the cysts per gram feces released by each individual mouse. Positive mice are those in which a cyst was seen to indicate infection, but the cyst was off of the haemocytometer grid and could not be counted.

	Days After Infection			
mouse	2	3	4	5
MALE				
1	0	0	0	2856000
2	0	0	65000	811000
3	0	0	88000	194000
4	0	2000	3000	197000
5	0	positive	62000	2000000
6	no feces	no feces	no feces	no feces
7	0	no feces	0	positive
8	0	0	0	3271000
9	0	0	no feces	3162000
10	0	0	15000	752000
11	0	3000	39000	2774000
FEMALE				
1	0	0	0	414000
2	0	0	0	48000
3	0	0	0	349000
4	0	0	0	914000
5	0	0	15000	762000
6	0	0	0	4266000
7	0	0	11000	400000
8	0	0	0	247000
9	0	0	0	1174000

Table 3-3. Cyst release after challenge (second) infections in male and female C57BL/6 mice. Values are the Mean ± SEM of the number of cysts per gram feces for all hosts examined. Mice had been previously infected for 60 days with *G. muris* and then drug cured.

Days after challenge	Male	#positive/ #examined	Female	#positive/ #examined
2	5000 ± 3000	3/20	10000 ± 9000	2/15
4	7000 ± 6000	2/19	0	0/15
6	49000 ± 27000	7/20	0	0/15
8	32000 ± 23000	4/17	0	0/15
10	14000 ± 12000	3/16	0	0/15

mean ratios and unequal variances in the raw data so the data was log transformed (log x + 1) prior to statistical analysis.

Trophozoite numbers during the primary infection were presented two ways (Fig. 3-3). The figures of abundance and intensity data showed a similar pattern. Males had significantly more trophozoites than females earlier in the infection and were still infected on day 50 when the females examined were negative for trophozoites.

A two-way analysis of variance of the abundance data showed significant effects of both time, gender and time X gender (all $P< 0.0001$; Table 3-4). Male mice had significantly greater ($P< 0.05$, one way ANOVA) numbers of trophozoites in their small intestine than females on days 10, 20 and 50 after infection (Figure 3-3). Females had a large decrease in the numbers of trophozoites on day 20 corresponding to the decrease in numbers of cysts released in the feces at this time. Males also had a decrease in the number of trophozoites but not to the same extent as females. In one of two experiments no observable trophozoites were seen in the female mice (N=5) on day 20. After day 20 both males and females showed fairly constant numbers of trophozoites but with females having no observable trophozoites on day 50. The increase in prevalence of cyst release on day 35 in the male mice was not observable in the numbers of trophozoites although, in the ten day interval between counts, a brief rise and fall may have been missed.

The analysis of the intensity data also showed a significant difference ($P< 0.001$, two-way ANOVA; Table 3-4) between the males and females and a significant effect of infection. However, the analysis of the abundance data showed a significant interactive effect among the two variables while the intensity data did not. Twenty days after infection many females did not have observable trophozoites in their intestines. This makes mean values of abundance look like there is a more dramatic difference in the numbers of trophozoites found between males and females. However, when this is examined only in positive animals the numbers of trophozoites found are closer between males and females. Thus, while males have more trophozoites than females, they are responding over time in a similar pattern.

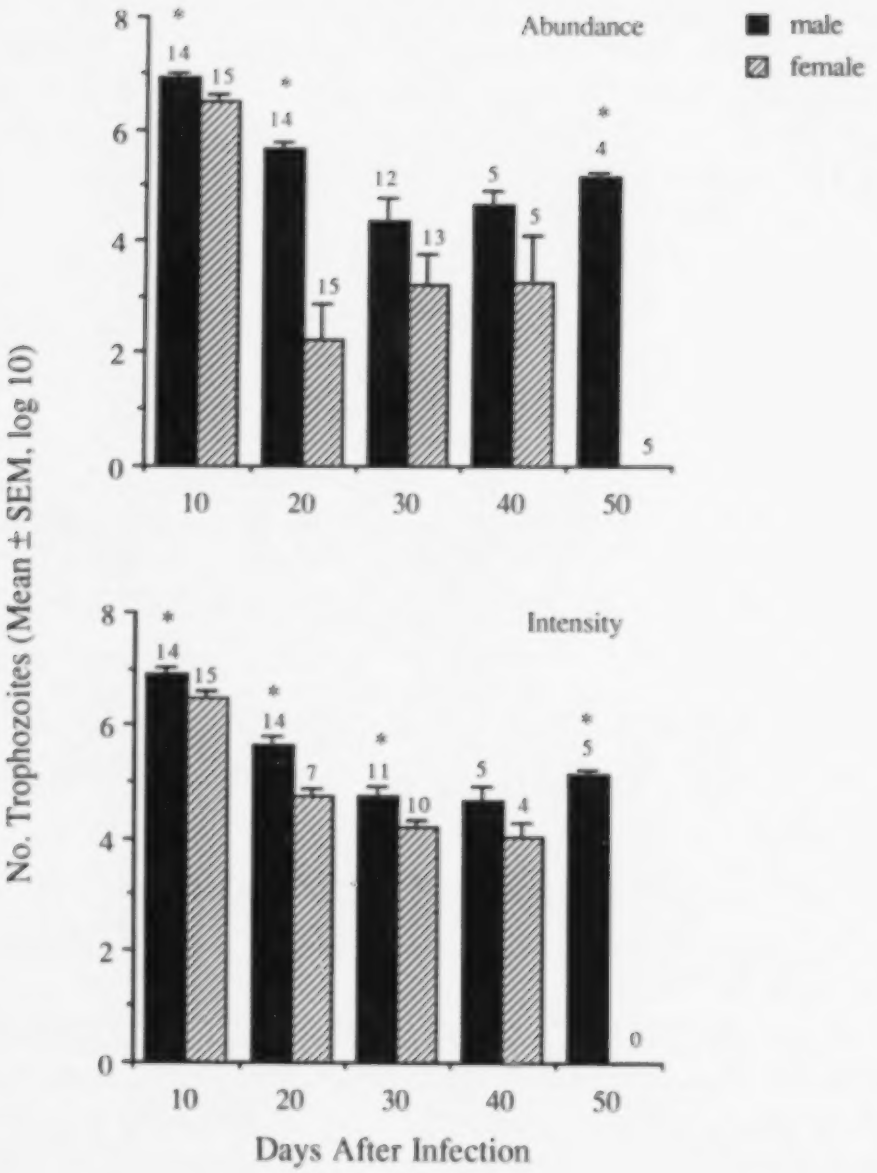

Figure 3-3. Trophozoite numbers in the small intestine of male and female C57BL/6 mice expressed as either the abundance or intensity of infection. The values are the geometric mean ± SEM of the number of trophozoites. The number above each bar is the sample size for each group. Asterisks represent significant differences between males and females at that time period ($P<0.05$, one-way ANOVA).

Table 3-4. Summary statistics for two-way ANOVA comparing gender and time after infection for trophozoite numbers in the small intestine of male and female C57BL/6 mice during a primary *Giardia muris* infection.

Abundance

ANOVA Table for log troph #

	DF	Sum of Squares	Mean Square	F-Value	P-Value
TIME	4	203.339	50.835	27.305	<.0001
GENDER	1	106.568	106.568	57.241	<.0001
TIME * GENDER	4	63.226	15.807	8.490	<.0001
Residual	93	173.141	1.862		

Intensity

ANOVA Table for log troph #

	DF	Sum of Squares	Mean Square	F-Value	P-Value
TIME	3	78.094	26.031	126.990	<.0001
GENDER	1	6.030	6.030	29.416	<.0001
TIME * GENDER	3	.689	.230	1.121	.3463
Residual	72	14.759	.205		

Trophozoite levels after a challenge infection with 1000 cysts were examined ten days after challenge. No trophozoites were observed in the intestines of eight male mice examined. One remaining male mouse was then treated with the immunosuppressant Dexamethasone (AZIUM-SP, Schering Canada Inc., Point-Claire, Quebec) for two days and the small intestine examined for trophozoites but none were observed. If there were low numbers of trophozoites in the intestine originally, this treatment should have allowed their numbers to increase to detectable levels (Nair *et al.*, 1981). The feces of these male and female mice were examined every two days after challenge (as described above) until they were killed on day 10. Unlike the males in the previous experiments, the males in this experiment released no cysts in their feces except for one male mouse in which I observed one cyst in his feces two days after challenge. It is possible that this cyst may have been from the challenge inoculum and passed through the intestine without excysting. Females had no detectable cysts in their feces. Two of eight female mice examined were positive for trophozoites with 10,000 and 27,000 trophozoites observed in the two mice.

DISCUSSION

The course of infection in female C57BL/6 mice, as measured by cyst release in feces, is similar to that reported by Belosevic *et al.* (1984) and Snider and Underdown (1986). The numbers of trophozoites in the intestine from three to five weeks after infection was higher in the study by Snider and Underdown (1986) (log four to five as opposed to log two to four in this study) but the females in their study had equivalent numbers of trophozoites early in the infection and had eliminated the parasites by week seven. The course of infection in male C57BL/6 mice has not been previously reported.

While the pattern of a similar acute infection followed by a longer infection in the males is similar to the study of Faubert *et al.* (1985) the increase in the prevalence of cyst release by the males later in the infection (day 33-35) has not been previously reported. The number of trophozoites observed at day 40 was not higher than on day 30, however, the numbers of trophozoites could have risen and fallen again within this time period.

While the cyst release in the feces on day 10 after infection was equivalent in the male and female mice, the males had significantly more trophozoites than females, indicating the males' lesser ability to control parasite numbers.

The cyst release data would suggest that females are not infected for more than approximately 20 days. However, the data on trophozoite levels in the small intestine do not support this conclusion. Males and females do not have significantly different numbers of trophozoites on day 40 after infection and both have consistently high numbers of trophozoites (above log four) for the first 40 days of the infection. On day 50 after infection the females did not harbor trophozoites in their intestines while the males were still infected. The puzzling question is why do males but not females release cysts later in the infection (from days 20 to 40) when they both have high numbers of parasites in the intestine? Two possibilities suggest themselves: 1) that the males' higher mean numbers of parasites are just enough to permit cyst formation or 2) that conditions in the small intestine, either immunological or physiological, are suitable for cyst formation in males but not females.

Cyst release data is seen as a good indicator of the level of infection during the acute phase of the infection (Roberts-Thomson *et al.*, 1976; Owen *et al.* 1979; Belosevic and Faubert, 1983a) but does not reflect, in this study, the differences between males and females in numbers of trophozoites in the intestine, ten and twenty days after infection. Olveda *et al.* (1982) also mentioned the inconsistent correlation between cyst output and trophozoite loads early and late in infection when no cysts are shed in the feces but significant numbers of trophozoites are present in the intestine. Cyst release in the feces, therefore, must be evaluated with caution and used only to measure broad general trends in infection.

The lack of observable trophozoites after challenge infections in males was puzzling. I have observed trophozoites in the small intestine of C57BL/6 males on previous occasions after challenge infections. As well, cyst release of male mice after challenge is normally greater than that observed with the males examined for trophozoites. As two of the females were positive for trophozoites I know that the mice were given an infectious dose of parasite so something else was different in this case. The cyst release data in challenge infections point to the males' lesser ability to

resist *Giardia* infections; however, more work needs to be done to clarify this point. When females of different strains are compared after challenge infections, they are all able to resist becoming reinfected, whether they are considered susceptible or resistant in a primary infection (Belosevic and Faubert, 1983b; Underdown *et al.*, 1981). The females in this study showed responses similar to those of other female mice studied.

Male C57BL/6 mice, then, establish a patent infection earlier than females of the same strain, have higher levels of parasites during the early stages of the infection and possibly eliminate the infection later than females. The females are generally refractory to a challenge infection while many males become reinfected and shed cysts. If parallels can be drawn to natural situations this could mean that previously exposed males, as well as naive animals, could harbor the parasite for long periods in a population and help insure persistence in an area. While female mice of many strains are able to eliminate the parasite and resist reinfection (Underdown *et al.*, 1981), it has been shown that apparently infection free female mice (but previously infected) will regain a patent infection if they become pregnant (Stevens and Frank, 1978), suggesting pregnant females could be another source of parasite persistence in the environment.

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Chapter Four: Serum antibody responses and parasite antigen recognition by male and female C57BL/6 mice infected with *Giardia muris*.

INTRODUCTION

The humoral components of the immune system are thought to play a major role in the elimination of *Giardia* from the host (see review in Chapter 2, this thesis). Mice deficient in all antibody classes after anti-IgM treatment from birth have chronic infections with *G. muris* (Snider *et al.*, 1985) and B cell deficient mice expressing the *xid* gene are also more susceptible (Snider *et al.*, 1988). As well, hypogammaglobulinemic humans have increased prevalence and severity of infection with *G. lamblia* (Ament and Rubin, 1972).

Serum from immunized mice will agglutinate trophozoites of *G. muris* and will cause lysis in the presence of complement (Belosevic and Faubert, 1987). Monoclonal antibodies (MAb) are able to fix complement and kill *G. muris* trophozoites *in vitro* (Heyworth, 1992) and will agglutinate trophozoites of *G. lamblia* (Guy, 1989; Heyworth *et al.*, 1989). Antibody can also be cytotoxic to *G. lamblia* in the absence of complement (Nash and Aggarwal, 1986). Butscher and Faubert (1988) demonstrated that an intra-peritoneal injection of infected mice with an anti-*G. muris* IgM MAb lessened parasite burden in the small intestine, although they did not determine the method of action. These MAb's were able to agglutinate trophozoites and to kill them in the presence of complement. Therefore, the available evidence indicates that anti-parasite antibody and complement may participate in host defense against *Giardia*.

While differential antibody responses between males and females exist in many parasitic infections (reviewed by Alexander and Stimson, 1988; Brabin and Brabin, 1992) no information is available concerning differential responses in male and female mice infected with *G. muris*. In human infections with *G. lamblia*, female patients have initially higher antibody titres (measured against all serum immunoglobulins but primarily to IgG) than male patients but these differences are not apparent more than 60 days after infection (Jokipii *et al.*, 1988).

In this chapter I examined parasite-specific serum antibody levels in males and females and examined whether differential antigen recognition occurs in the mice. The measurements of the three main antibody classes,

IgM, IgA and IgG, were done at three time periods during the primary infection and after challenge infections. The time periods in the primary infection (at 10, 20 and 40 days after infection) correspond to the time of peak parasite numbers, decline and chronic infection periods. Mice have four IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3) with different physical properties and actions (Roitt *et al.*, 1985). For this reason, measurements of these IgG subclasses were done at the same time. The analysis of parasite protein recognition by serum IgG was done using a Western blot technique where separated parasite antigens are bound to nitrocellulose sheets and reacted with infected sera. This was an important experiment to do as the mice may recognize different parasite antigens even if their IgG levels are similar. A comparison of antigen recognition by susceptible and resistant mice infected with *G. muris* has shown differences in their ability to recognize some parasite antigens (Erlich *et al.*, 1983).

MATERIALS AND METHODS

Mice

Specific pathogen free (SPF) eight-week old male and female C57BL/6 mice were used in all experiments. The mice were purchased from Taconic Laboratories (Germantown, N.Y.) and maintained under SPF conditions throughout the study.

Parasite

The *Giardia muris* used in the experiments was initially isolated from a Golden hamster by Roberts-Thomson *et al.* (1976). The parasite was maintained in the laboratory by passages of 10,000 cysts/mouse administered by gavage to CD-1/CRBr mice. Passages were done every two weeks.

Collection of sera

Sera were collected from mice by cardiac puncture. The mice were killed by cervical dislocation and then bled with a 1 cc syringe fitted with a 23 gauge needle. The blood was placed in 1.5 ml snap cap tubes, left at

room temperature for less than 5 min and then placed at 4° C for one to two hours. The samples were then centrifuged at 3000 X *g* for 10 min in a microcentrifuge (MSE Micro-Centaur, Johns Scientific), the serum removed with a Pasteur pipet, and frozen at -20° C until assayed. Pooled serum was also collected for use in immunoblotting. Groups of males or females were decapitated, the blood collected in a tube and then treated as above.

Isolation of trophozoites from the small intestine

To collect trophozoites, the upper half of the small intestine was removed, slit longitudinally, and cut into 3 cm pieces. The segments of the small intestine were suspended in phosphate buffered saline (PBS) and agitated with a stirring rod for 30 min at room temperature. The suspension was strained through 20 layers of cheesecloth, centrifuged at 600 X *g* for 10 minutes and the pellet resuspended in RPMI 1640 culture medium (Gibco, Grand Island, NY) containing 20% fetal bovine serum (Gibco). The solution containing trophozoites was incubated at 37°C in glass Petri dishes to allow the parasites to adhere to glass. The supernatant was decanted, PBS added to the dishes, and trophozoites were removed by placing the Petri dishes on ice for 10 min. Isolated trophozoites were washed once in PBS by centrifugation at 600 X *g* and stored at -20°C prior to preparation of the soluble extract. Trophozoites were collected from groups of CD-1 mice infected for seven days with *G. muris*.

Preparation of soluble trophozoite extract

Trophozoites isolated from the small intestine of mice were sonicated for 5 min with a Kontes (Vineland, NJ) Micro-Ultrasonic Cell Disrupter at maximum output. This material was then centrifuged for 5 min at 3000 X *g* and the pellet discarded. Protein concentration of the extract was determined using the Bio-Rad Protein Determination Kit (Bio-Rad , Richmond, Ca.).

Assay for serum antibodies

An enzyme-linked immunosorbent assay (ELISA) was performed as described by Voller *et al.* (1976) to measure the levels of anti-parasite specific antibody in the serum. In all ELISA's the wells of 96 well polystyrene plates (Immulon II, Dynatech) were coated with 100 µl of a 10

µg/ml solution of trophozoite protein suspended in PBS and incubated overnight at 4º C. The plates were then washed once with PBS and areas of the wells not coated with parasite protein blocked for one hour at room temperature (RT) with 150 µl of blocking buffer (PBS with 1% bovine serum albumin and 0.1% Tween-20) to prevent non-specific binding. Plates were washed 3 times with washing buffer (PBS with 0.1% Tween-20), using a plate washer (Nunc-Immuno Wash 12), after each step except as noted.

Total serum IgG was measured by adding a 1/100 dilution of mouse serum, diluted in blocking buffer, to the plates and incubating for one hour at RT. The conjugate solution (anti-mouse IgG conjugated to horse radish peroxidase (HRP), SIGMA) was added at a 1/1000 dilution, diluted in blocking buffer , and incubated for one hour at RT. The plate was then washed three times with washing buffer. To develop the colour, 100 µl of 2,2'-azino-di- [3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) (Peroxidase Substrate Kit; Bio-Rad) was added to the wells and incubated for 30 min. The colour reaction was quantified at 405 nm using an automated microplate reader (Bio-Tek Instruments Inc., Highland Park, VT).

Measurements of parasite specific IgA, IgM, and IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b} and IgG_3) were done as above except that the sera were diluted 1/50 and the antibodies recognized by rabbit antisera against these classes. The antisera were obtained from the Mouse Typer ® Sub-Isotyping Kit (Bio-Rad). The conjugate used was a goat anti-rabbit IgG HRP (Sigma) at a 1/3000 dilution.

All values from the ELISA's are expressed as the mean optical density of duplicate samples.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of parasite proteins

The method of Laemmli (1970) was used to perform the SDS-PAGE separation of parasite proteins. The proteins were first heated at 95º C for 5 min in reducing sample buffer and then electrophoresed on Bio-Rad MINI-Protean II slab gels. The samples were electrophoresed at 100 V through a 4% stacking gel and then at 200 V through a 5 to 12% gradient gel. The runs typically took one hour. Ten µg of parasite protein was analyzed in each well and high and low molecular weight standards (Bio-Rad) were

separated on each gel for molecular weight size comparison. Gels were stained for one half hour with Coomassie brilliant blue, destained in methanol-acetic acid and then silver stained using the Bio-Rad silver staining kit.

Immunoblotting

The parasite antigens recognized by serum IgG were determined by Western blot using the method of Towbin *et al.* (1979). After SDS-PAGE separation the proteins were electrophoretically transferred to a nitrocellulose sheet using a Tris glycine methanol buffer system (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). The proteins were transferred in a Bio-Rad Mini Trans-Blot® Electrophoretic Cell for one hour at 100 V. Starting temperature of the cell and contents was approximately 4° C and end temperature approximately 25° C. Biotinylated high and low molecular weight standards (Bio-Rad) were analyzed on each gel. The nitrocellulose sheet was removed at the end of the transfer, cut into strips, and placed in blocking buffer (25mM Tris-HCl, 150mM NaCl, and 1% skim milk powder, pH 7.5) for at least one hour at RT.

The strips were washed twice with Tris buffered saline with 0.05% Tween-20 (TTBS: 20mM Tris, 500mM NaCl, pH 7.5) and the sera added to each well at a 1/50 dilution in blocking buffer. The strips were incubated overnight at 4° C, washed twice with TTBS and the antibody conjugate (goat-anti-mouse alkaline phosphatase; Bio-Rad) added at a 1/3000 dilution. The conjugate was incubated for 1.5 hours at RT. Unbound antibody was removed by two washes with TTBS. The Tween was removed from the membranes with a final wash of Tris buffered saline. The colour development solution (containing 5-bromo-4 chloro-3-indolyl phosphate and nitroblue tetrazolium; Bio-Rad) was added to the strips and incubated for 15 to 30 minutes at RT. Colour development was stopped by washing the strips in distilled water.

Strips containing the molecular weight standards were treated as above, including incubation with uninfected mouse serum, except that avidin-alkaline phosphatase conjugate (Bio-Rad) was substituted for the antibody conjugate during that step in the process.

Experimental design

In all experiments mice were infected on day 0 with 1000 cysts each of *G. muris*. During the primary infection, sera were collected from 5 infected and 5 uninfected mice (of each sex) on days 10, 20 and 40 after infection. Challenge mice were treated with 1 mg metronidazole each for three consecutive days after the primary infection (days 60-63) to eliminate the infection, rested for 10 days, and then challenged. Challenge serum samples were collected 10 days after challenge.

Challenge infections were done with 6 to 9 mice per group. Groups consisted of uninfected mice, mice which had been previously infected and drug cured but not challenged (controls), groups challenged with 1000 cysts, and groups challenged with 50 μg of trophozoite extract.

RESULTS

SERUM ANTIBODY LEVELS

A. Primary infection:

During the primary infection with *G. muris* in the male and female mice there was a significant increase in the levels of IgM in both sexes and of IgG in females, but not males. Levels of parasite specific IgM (Fig. 4-1) were significantly affected by both infection and time ($P < 0.001$, Three way ANOVA; Table 4-1) and showed a significant interactive effect between these two variables. There was no effect of gender. Levels of serum IgA were affected by time and the interaction between time and infection but showed no other effects. The levels of serum IgG were significantly affected by infection, gender, time and several other interactive effects (Table 4-1). However, only the females showed any significant increases in IgG levels on days 20 and 40 (one way ANOVA; Fig. 4-1) while males had no significant increases in IgG when compared to controls.

An examination of the levels of IgG subclasses showed no marked differences in any of the four subclasses 10 days after infection (one way ANOVA; Fig. 4-2). A three way ANOVA (Table 4-2) showed little overall effects present in the IgG_1 and IgG_{2a} subclasses. There was a significant effect of infection on IgG_1 levels while a significant interactive effect between infection and gender was present in the IgG_{2a} subclass. This

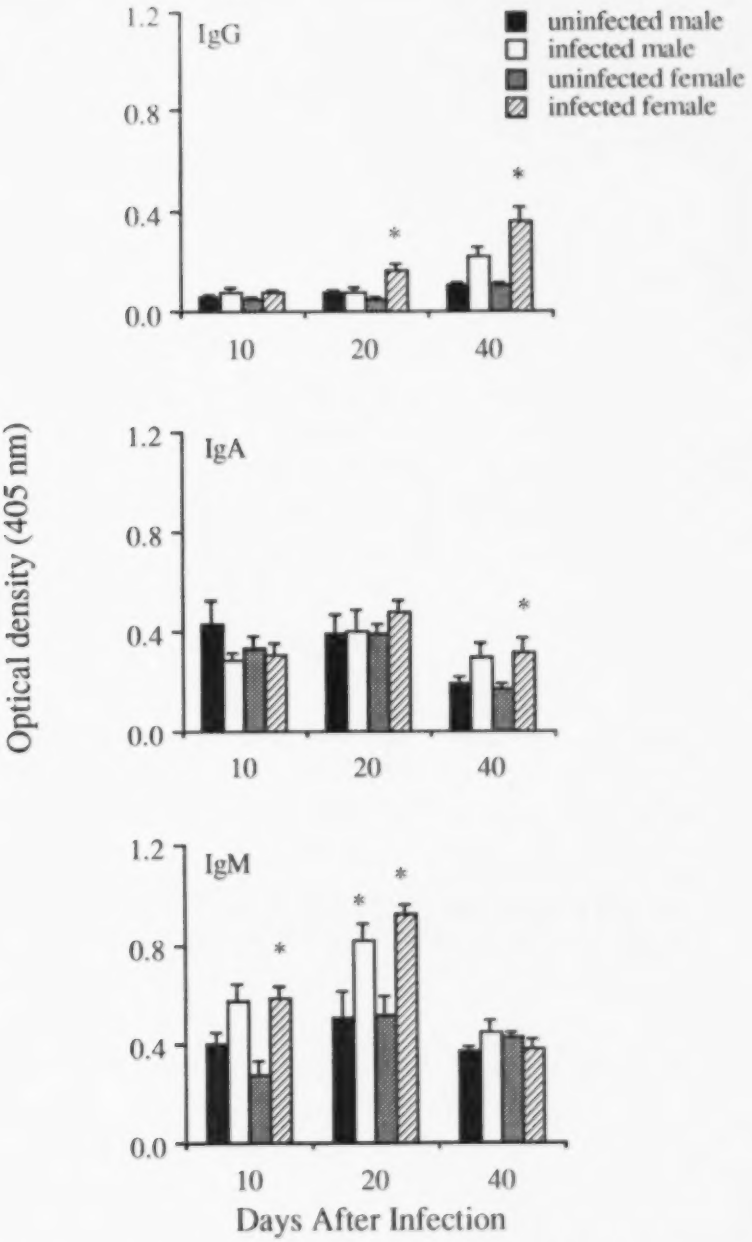


Figure 4-1. Parasite specific serum antibody levels of IgG, IgA and IgM in male and female mice during a primary *Giardia muris* infection. Results are the mean optical density ± SEM, measured by ELISA, of 5 mice per group. Infected females on day 20 and uninfected males on day 40 had N=4. Asterisks represent significant differences ($P<0.05$, one-way ANOVA) between that group and the other group of the same sex.

Table 4-1. Summary statistics for three-way ANOVA comparing infection, time and gender for serum levels of IgG, IgA and IgM during a primary *Giardia muris* infection.

ANOVA Table for IgG

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	.111	.111	41.106	<.0001
Gender	1	.015	.015	5.586	.0224
Infection * Gender	1	.030	.030	11.051	.0017
Time	2	.192	.096	35.575	<.0001
Infection * Time	2	.065	.032	11.989	<.0001
Gender * Time	2	.015	.007	2.698	.0780
Infection * Gender * Time	2	.014	.007	2.558	.0884
Residual	46	.124	.003		

2 cases were omitted due to missing values.

ANOVA Table for IgA

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	.012	.012	.773	.3837
Gender	1	4.267E-6	4.267E-6	2.651E-4	.9871
Infection * Gender	1	.020	.020	1.244	.2703
Time	2	.284	.142	8.816	.0005
Infection * Time	2	.112	.056	3.474	.0390
Gender * Time	2	.012	.006	.379	.6869
Infection * Gender * Time	2	.004	.002	.127	.8814
Residual	48	.773	.016		

ANOVA Table for IgM

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	.588	.588	34.205	<.0001
Gender	1	1.918E-4	1.918E-4	.011	.9164
Infection * Gender	1	.004	.004	.254	.6167
Time	2	.863	.431	25.070	<.0001
Infection * Time	2	.283	.142	8.225	.0009
Gender * Time	2	.031	.016	.913	.4083
Infection * Gender * Time	2	.045	.022	1.301	.2822
Residual	46	.791	.017		

2 cases were omitted due to missing values.

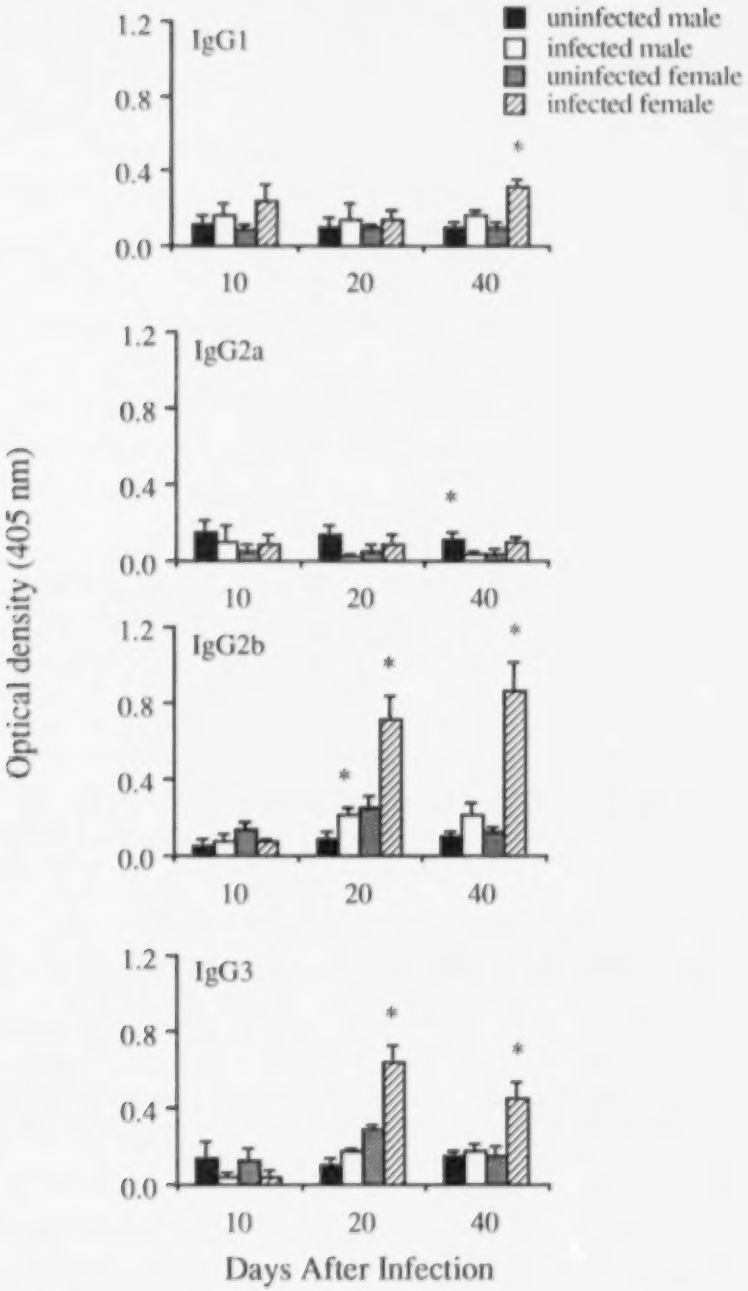


Figure 4-2. Parasite specific serum levels of IgG subclasses in male and female mice during a primary *Giardia muris* infection. Results are the mean optical density ± SEM, as measured by ELISA, of n=5 mice per group. Asterisks represent significant differences ($P<0.05$, one-way ANOVA) between that group and the other group of the same sex.

Table 4-2. Summary statistics for two-way ANOVA comparing infection, gender and treatment for serum levels of four IgG subclasses during a primary *Giardia muris* infection.

ANOVA Table for IgG1

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	.121	.121	9.706	.0031
Gender	1	.019	.019	1.496	.2272
Infection * Gender	1	.030	.030	2.371	.1302
Time	2	.024	.012	.953	.3926
Infection * Time	2	.024	.012	.964	.3887
Gender * Time	2	.016	.008	.645	.5291
Infection * Gender * Time	2	.017	.008	.680	.5117
Residual	48	.600	.012		

ANOVA Table for IgG2a

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	.005	.005	.493	.4860
Gender	1	.007	.007	.766	.3859
Infection * Gender	1	.057	.057	5.860	.0193
Time	2	.006	.003	.318	.7290
Infection * Time	2	.003	.001	.147	.8640
Gender * Time	2	.005	.003	.274	.7615
Infection * Gender * Time	2	.002	.001	.090	.9139
Residual	48	.466	.010		

ANOVA Table for IgG2b

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	.819	.819	37.753	<.0001
Gender	1	.842	.842	38.794	<.0001
Infection * Gender	1	.304	.304	14.024	.0005
Time	2	.744	.372	17.150	<.0001
Infection * Time	2	.524	.262	12.079	<.0001
Gender * Time	2	.292	.146	6.723	.0027
Infection * Gender * Time	2	.306	.153	7.062	.0020
Residual	48	1.042	.022		

ANOVA Table for IgG3

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	.129	.129	8.525	.0053
Gender	1	.357	.357	23.579	<.0001
Infection * Gender	1	.126	.126	8.294	.0059
Time	2	.472	.236	15.574	<.0001
Infection * Time	2	.245	.122	8.083	.0009
Gender * Time	2	.271	.136	8.949	.0005
Infection * Gender * Time	2	.058	.029	1.918	.1580
Residual	48	.727	.015		

indicates that, while gender and infection are not independently being affected, the males and females were responding differently. In contrast, IgG_{2b} and IgG_3 had very significant effects of all the variables examined except that IgG_3 did not have a significant interaction between infection, gender and time. These interactions were strongly influenced by the greater increase in the levels of these two subclasses in the female mice and by the increase in levels of IgG_{2b}, but not IgG_3, in the males (Fig. 4-2).

Thus, the primary infection in the male and female mice was characterized by a strong, but equal IgM response and by significant increases in IgG levels in female but not male mice. The increases of IgG were not made up equally of the IgG subclasses but were primarily due to IgG_{2b} and IgG_3.

B. Antibody levels after challenge infection

After challenge infections there were no significant changes in IgM levels (Fig. 4-3) either between males and females or with treatment (Table 4-3). However, both IgG and IgA (Fig. 4-3) had significant differences between males and females ($P< 0.05$, Two way ANOVA; Table 4-3). The two-way ANOVA's compared differences between the controls (old infected) and the challenge groups. The challenge groups all showed an increase over uninfected mice. The females had significantly higher antibody levels of IgG and IgA than the males after a challenge with cysts but not after challenge with the trophozoite extract.

After challenge, the treatments had no significant effect on the four IgG subclasses (Fig. 4-4) ($P< 0.05$, Two way ANOVA; Table 4-4). Only IgG_{2b} levels were significantly affected by gender and gender X treatment. A comparison of means in the groups showed that, while no difference exists between uninfected mice, females had significantly higher levels of IgG_{2b} than males in all three challenge groups. Comparisons among the groups also showed no significant difference between any of the groups of males.

SDS-PAGE analysis of proteins and immunoblotting.

The SDS-PAGE analysis of the parasite proteins showed a large number of bands on the gel ranging in size from approximately 10 to 250

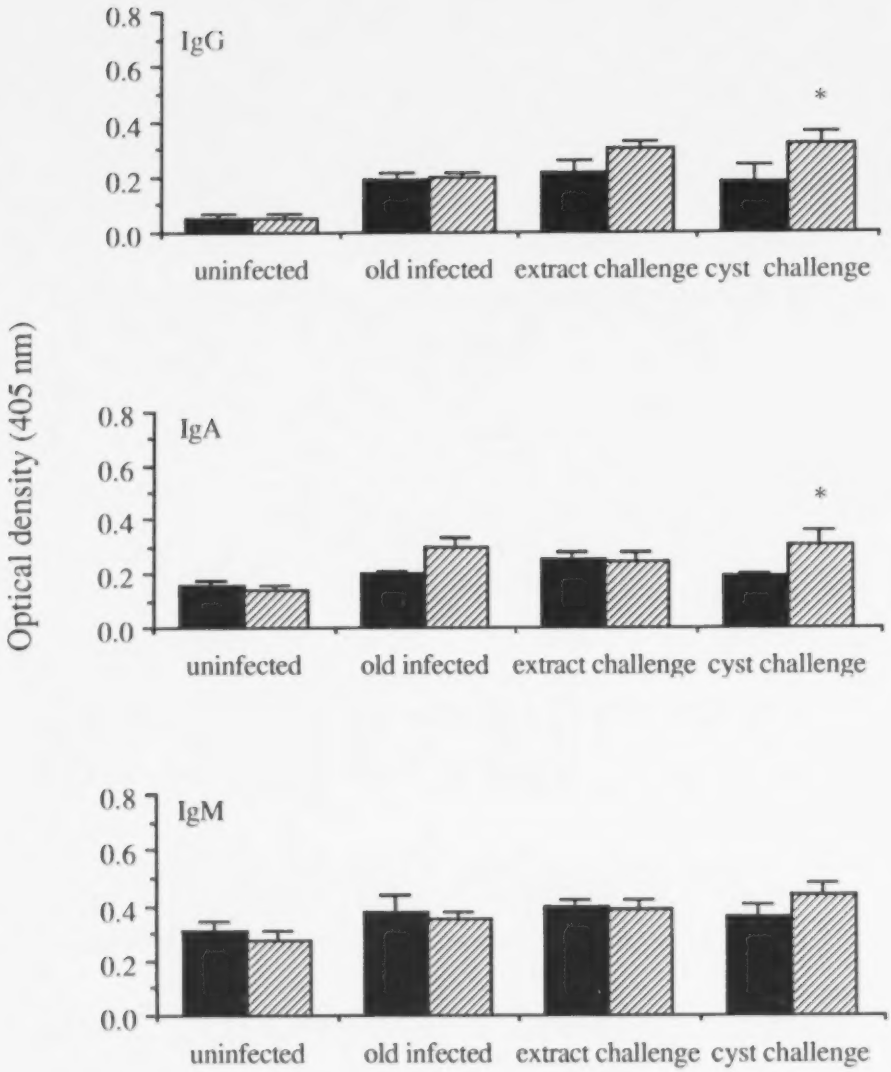


Figure 4-3. Parasite specific serum antibody levels of IgG, IgA and IgM in male and female mice 10 days after challenge infections with either cysts or trophozoite extract of *Giardia muris*. Results are the mean optical density ± SEM, as measured by ELISA, of n= 6 to 9 mice per group. Solid bars are males and hatched bars are females. Asterisks represent significant differences ($P<0.05$, one-way ANOVA) between that group and the other group of the same sex.

Table 4-3. Summary statistics of two-way ANOVA comparing variation among gender and treatment for serum levels of IgG, IgA and IgM after challenge infections with *Giardia muris*. Comparisons were made between old infected mice and the two challenge groups.

ANOVA Table for IgG

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	.034	.034	5.773	.0273
Treatment	2	.021	.011	1.812	.1918
Gender * Treatment	2	.018	.009	1.516	.2464
Residual	18	.105	.006		

ANOVA Table for IgA

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	.058	.058	7.737	.0081
Treatment	2	5.355E-5	2.677E-5	.004	.9964
Gender * Treatment	2	.033	.016	2.176	.1264
Residual	41	.309	.008		

ANOVA Table for IgM

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	.003	.003	.230	.6340
Treatment	2	.010	.005	.383	.6842
Gender * Treatment	2	.026	.013	.951	.3949
Residual	41	.552	.013		

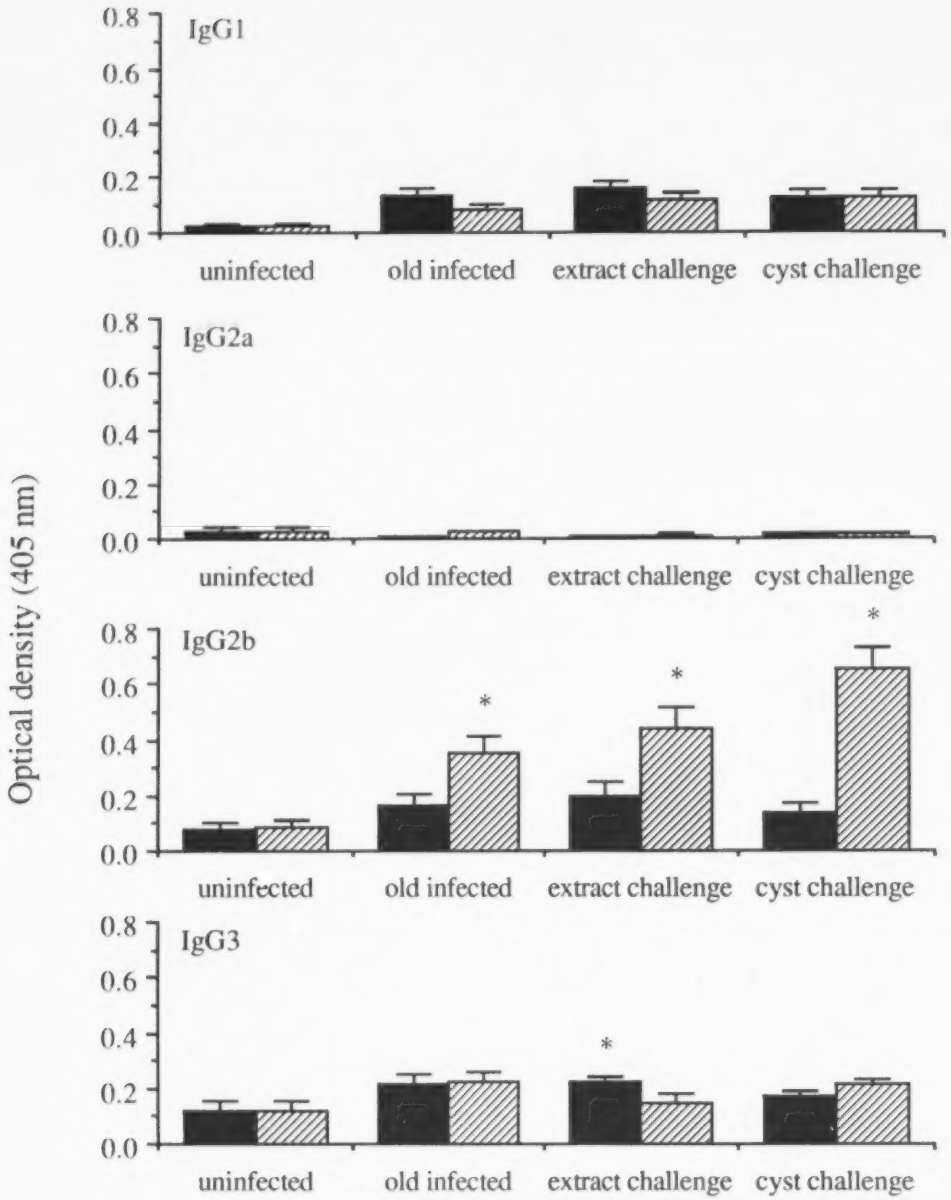


Figure 4-4. Parasite specific serum levels of IgG subclasses in male and female mice after challenge infections with *Giardia muris*. Results are the mean optical density ± SEM, as measured by ELISA, of n=6 to 9 mice per group. Solid bars are males and hatched bars are females. Asterisks represent significant differences ($P<0.05$, one-way ANOVA) between that group and the other group of the same sex.

Table 4-4. Summary statistics for two-way ANOVA comparing variation among gender and treatment for serum levels of four IgG subclasses after challenge infections with *Giardia muris*. Comparisons were made between old infected mice and the two challenge groups.

ANOVA Table for IgG1

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	.015	.015	3.486	.0690
Treatment	2	.006	.003	.707	.4990
Gender * Treatment	2	.006	.003	.645	.5302
Residual	41	.179	.004		

ANOVA Table for IgG2a

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	4.937E-4	4.937E-4	1.706	.1988
Treatment	2	2.625E-4	1.313E-4	.454	.6385
Gender * Treatment	2	.001	2.574E-4	.889	.4187
Residual	41	.012	2.894E-4		

ANOVA Table for IgG2b

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	1.151	1.151	41.097	<.0001
Treatment	2	.157	.078	2.798	.0726
Gender * Treatment	2	.250	.125	4.462	.0177
Residual	41	1.148	.028		

ANOVA Table for IgG3

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	.001	.001	.161	.6903
Treatment	2	.008	.004	.716	.4945
Gender * Treatment	2	.026	.013	2.194	.1244
Residual	41	.242	.006		

kDa (Fig. 4-5).

An examination of the blots exposed to pooled serum taken at intervals over the course of the infection showed no recognition of antigens by either control serum or serum collected from mice infected for 10 days. Twenty days after infection two weak bands (approximately 68 and 59 kDa in weight) and several strong bands around 30 kDa were present (Fig. 4-6A). These proteins below 31 kDa were strongly recognized by both male and female mice and by 50 days after infection at least 5 proteins between 27 and 33 kDa were recognized. More weakly recognized were two bands of 43 and 47 kDa and three bands at 58-68 kDa. Serum from males infected for 50 days did weakly recognize two bands at 166 and 184 kDa that were not recognized by the pooled serum from females or in the individual females examined.

An examination of sera from four males and four females infected for 40 days showed the same bands recognized by the pooled serum, however, some bands were more strongly recognized by some individuals and some mice did not appear to recognize all of the proteins recognized by the pooled serum (Fig. 4-6B). The two high weight bands recognized only by the males in the pooled serum were recognized by one of the four individual males examined.

DISCUSSION

The strong IgM response was equal in both males and females and showed a typical early infection response to an infectious agent (Roitt *et al.*, 1985). This strong IgM response contradicts an earlier study using female C57BL/6 mice that found no anti-parasite serum IgM (Snider and Underdown, 1986).

The levels of IgA in the serum did not increase until later in the infection in both males and females. A similar late rise of serum IgA was reported by Snider and Underdown (1986). This suggests that IgA is not an important contributor to parasite control during infection. However, the early appearance of secretory IgA in the intestine and its possible role in parasite control has been well documented (Heyworth, 1986, 1989; Heyworth and Pappo, 1990). In this case the serum levels of IgA are obviously not representative of this antibodies' role during the course of the infection.

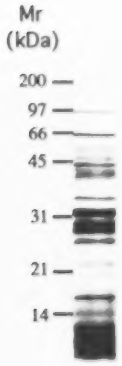
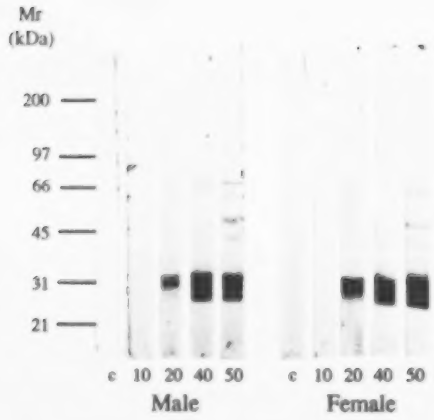


Figure 4-5. SDS-PAGE analysis of trophozoite extract of *Giardia muris*. The gel has been silver stained. M_r = molecular weight.

A



B

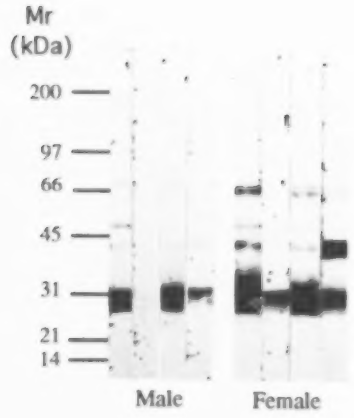


Figure 4-6. Western blot analysis of (A) IgG antibodies in sera of *Giardia muris* infected male and female mice from pooled serum sources on 0 (control), 10, 20, 40 and 50 days after infection (B) IgG antibodies from four individual male and four individual female mice infected for 40 days.

An increased serum IgG level beginning 14 days after infection with *G. muris* was found in C57BL/6 females (Snider and Underdown, 1986). This corresponds to my findings of significant increases in the levels of IgG in females between 10 and 20 days after infection. The females, during the primary infection and after challenge, had higher IgG antibody levels than the males suggesting that the ability of females to mount a humoral immune response to the parasite is much greater than that of males.

The IgG subclasses have not been previously examined in a *G. muris* infection or any other intestinal extracellular protozoan parasitic infection. My results show a strong IgG_{2b} and IgG_3 response to the parasite during the primary infection and that females have a much stronger response than males. The IgG_3 subclass is normally present in the smallest amounts in mouse serum (Grey *et al.*, 1971) but is strongly produced in response to carbohydrate antigens, especially those of bacteria (Perlmutter *et al.*, 1978). Specific anti-parasite IgG_3 monoclonal antibodies are able to agglutinate trophozoites of *G. muris in vitro* (Heyworth, 1989) and to kill them in the presence of complement (Heyworth, 1992). The above, plus the fact that IgG_3 will cross the mouse placental barrier six times more readily than other isotypes (Grey *et al.*, 1971), suggest that IgG_3 may play an important role in intestinal immunity to bacterial and protozoan pathogens.

The levels of IgG_{2b} were elevated in both males and females during the primary infection, although much more strongly in the females. After the challenge infections, only IgG_{2b} increased. This increase was found only in the females and indicates, once again, that females respond more vigorously than males to the parasite antigens.

The strong increase in IgG_{2b} is interesting as this subisotype has been implicated in immune suppression of cell-mediated immune responses. Administration of aggregates of IgG_{2b} to spleen cell cultures from mice suppress antibody response to trinitrophenylated sheep red blood cells (Saito-Taki *et al.*, 1990), while administration of aggregates of IgG_1 and IgG_3 enhanced the response. This effect is due to an increase in prostaglandin E synthesis by the macrophages and lymphocytes in the cultures (Saito-Taki *et al.*, 1990). As well, peritoneal exudate cells treated with IgG_{2b} cause the activation of suppressor T cells when the peritoneal cells are injected into mice (Ptak *et al.*, 1988).

Suppression of the response to antigens has been demonstrated in *G. muris* infections in mice. Infected mice are less able to mount immune responses to T cell dependent antigens, and adherent peritoneal cells from these mice were less able to kill tumour-cell targets (Brett, 1983). Suppression of spleen cell antibody formation to sheep red blood cells also occurs in mice with an ongoing infection with *G. muris* (Belosevic *et al.*, 1985).

Thus, the increase in IgG_{2b} in the mice may not be for parasite control but for host suppression of certain immune responses. This suppression could be beneficial to the mice. The host immune response to intestinal parasites allows more molecules from the intestine to leak into the general circulation which could result in the development of severe allergies to food and the normal bacterial flora of the host. Such a detrimental response could result in the formation of immunosuppressive responses such as that caused by IgG2b.

The lack of IgG_1 and IgG_{2a} responses in the mice suggests two possibilities: 1) the antigens that best stimulate these subclasses are not present in sufficient quantities in *G. muris* , or 2) this protozoan parasite is not stimulating the class of T cells that are involved in the immunoglobulin class switch from IgM to other antibody classes. IgG_1 is produced in humans in response to polypeptide antigens (Stevens *et al.*, 1983). Different cytokines produced by T cells also affect the IgG subclass production. For example, IFN-γ stimulates an IgG_{2a} response and IL-4 induces an IgG_1 response (Thomson *et al.*, 1993).

The strong recognition of the bands around 30 kDa is similar to that reported in two other papers (Erlich *et al.*, 1983; Heyworth and Pappo, 1990) and suggests that these antigens are strongly immunogenic in this system. In a comparison of susceptible C3H/He males and resistant BALB/c females, the BALB/c mice better recognized a series of acidic 32 kDa antigens. Mice of both strains strongly recognized a series of antigens between 25 to 32 kDa (Erlich *et al.*, 1983). Similarly, a 30 kDa antigen was strongly recognized by intestinal IgA (Heyworth and Pappo, 1990). Erlich *et al.* (1983) also found a difference between the strains in the recognition of an 82 kDa antigen. In this study, I did not find that C57BL/6 mice recognized an antigen of this molecular weight .

G. lamblia trophozoites have several proteins of approximately 30 kDa, which include cytoskeletal and surface molecules (Crossley and Holberton, 1983; Crossley *et al.*, 1986). A surface lectin of *Giardia* of this same molecular weight has received considerable attention since its first report (Lev *et al.*, 1986). This molecule, termed taglin, is a surface membrane associated lectin which is activated by trypsin (Ward *et al.*, 1990). As this molecule may be associated with binding of trophozoites, or specifying their attachment site, it may be an important target for the host immune response. A similar lectin activity has also been identified in *G. muris* (Ward *et al.*, 1990). Therefore, surface cytoskeletal components and a lectin around 30 kDa would be important host targets for parasite control.

The individual mice in this study had noticeable differences in their recognition of most of the antigens other than those between 27 to 32 kDa. These individual differences overshadowed the differences between males and females and makes it difficult to say that recognition of any particular antigen is important in differential control of *G. muris*. However, in all of the blots, females had a stronger reaction to the higher weight proteins than males. This could indicate that the females have a greater antibody response than males. It could also be an indication that the IgG antibodies in the females have a higher affinity (binding strength) for the higher molecular weight antigens than do males. The pooled serum in both males and females had weaker recognition of these higher molecular weight proteins than did individual sera, so possibly these antibodies are being diluted out more in the pooled samples. It was, unfortunately, not possible to relate the individual sera responses to individual parasite loads in these mice. The trophozoite counts from other mice examined, however, show no significant differences at this time period (day 40) between males and females. Earlier in the infection, however, males do harbor more trophozoites in the intestine than females and this may influence the response of the individuals.

The infection data and the serum Ab responses indicate that females are more resistant to the parasite and produce a stronger IgG response than males. The strong IgM response was virtually identical in the males and females and gives no indication that this antibody is important in differential control of the parasite. In other studies, IgM can control parasite numbers *in vivo* (Butscher and Faubert, 1988) and is able to kill and

agglutinate trophozoites *in vitro* (Butscher and Faubert, 1988; Heyworth, 1992).

While differential antigen recognition may not be occurring between males and females there are very distinct differences in their production of IgG subclasses. The data in this chapter indicates that production of the IgG subclasses IgG_{2b} and IgG_3 may be important contributors in the differential control of the parasite seen between males and females. As well, the high levels of IgG_{2b} may be involved with suppression of host immune responses.

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Chapter Five: Inflammatory responses in male and female C57BL/6 mice infected with *Giardia muris*.

INTRODUCTION

Several lines of evidence suggest that inflammation plays a major role in the control of *G. muris* infections in mice. Mast cells release many mediators involved with inflammation and mast cell deficient W^f/W^f mice develop chronic *G. muris* infections (Erlich *et al.*, 1983). Treatment of resistant BALB/c mice with cyproheptadine, an inhibitor of the inflammatory mediators histamine and serotonin, causes prolonged infections (Erlich *et al.*, 1983). W^f/W^f mice also have a deficient eosinophil response (Vadas, M.A., unpublished, quoted by Mitchell *et al.*, 1982) and a stem cell defect leading to a macrocytic anemia (Russell and Bernstein, 1966). These defects could also lead to an impaired inflammatory response. C3H/HeN mice, which are susceptible to *G. muris*, are non-responsive to the histamine sensitizing factor of *Bordetella pertussis* (Bergman and Munoz, 1968). Intestinal *Trichinella spiralis* infections in mice, which cause a large intestinal inflammatory response, result in lower numbers of *G. muris* trophozoites in concurrent infections (Roberts-Thomson *et al.*, 1976a). Thus, the available evidence suggests that inflammation during *G. muris* infection may play a critical role in the elimination of this parasite from the small intestine.

The ability of mice to mount inflammatory responses is correlated with host genotype. Recruitment of thioglycollate-elicited peritoneal inflammatory cells is greater in resistant B10.A mice than in susceptible A/J mice during the acute and elimination phases of the infection with *G. muris*. However, mice of both strains recruit lower numbers of cells when infected (Belosevic and Faubert, 1986). As ongoing inflammatory events in one location suppress inflammation in other sites (Castro *et al.*, 1980; Normann *et al.*, 1991), this may be further evidence for inflammation occurring in the intestine during infection with *Giardia*.

In this chapter I described two aspects of inflammation; cellular responses and levels of inflammatory mediators. I compared the ability of male and female mice to recruit cells into the peritoneal cavity after an inflammatory stimulus and examined the types of cells recruited. The ability of the collected macrophages to phagocytose opsonized sheep red

blood cells *in vitro* was also examined. The levels of two potent mediators involved in inflammatory function and immune regulation were measured in serum. These were.tumour necrosis factor-alpha (TNFα) and interferon-gamma (IFNγ)

MATERIALS AND METHODS

Mice

Specific pathogen free (SPF) eight-week old male and female C57BL/6 mice were used in all experiments. The mice were purchased from Taconic Laboratories (Germantown, N.Y.) and maintained under SPF conditions throughout the study.

Parasite

The *G. muris* used in the experiments was initially isolated from a Golden hamster by Roberts-Thomson *et al.* (1976b). The parasite was maintained in the laboratory by passages of 10,000 cysts per mouse administered by gavage to CD-1/CRBr mice. Passages were done every two weeks.

A. PERITONEAL CELLS

Isolation and enumeration of cells

To induce an inflammatory response, mice were injected intraperitoneally with 1 ml of 3% Brewer's thioglycollate (Difco Laboratories, Detroit, MI). Three days after thioglycolate injection the mice were killed by decapitation and inflammatory cells collected from the peritoneal cavity by lavage using 10 ml of RPMI-1640 medium (Gibco Laboratories, Grand Island, N.Y.) with 10% fetal bovine serum (GIBCO) and 50 µg/ml gentamicin (GIBCO). Cells recovered were counted with a haemocytometer. The numbers of each cell type was determined by differential cell counts. Ten µl of the cell suspension was placed in a conical holder and centrifuged at 45 X *g* onto a glass microscope slide with a cytocentrifuge. The slides were stained with LeukoStat solutions (Fisher Scientific, Pittsburgh, PA) and differential counts of 200 cells

were made. Non-inflammatory, resident, peritoneal cells were collected and examined using the same procedure.

Opsonization of sheep red blood cells

Three ml of packed sheep red blood cells (SRBC) in Alsever's solution (Cedarlane, Hornby, ON) were washed twice by centrifugation in 10 ml of sterile phosphate buffered saline (PBS) at 400 X *g* for 10 min and the pellet resuspended to 10 ml in RPMI-1640 medium with 10% FBS and gentamicin. One ml of the SRBC preparation was added to one ml of a 1/100 dilution of rabbit anti-SRBC polyclonal serum (Cedarlane) and incubated for 30 min at 37° C with gentle shaking every 10 min. This preparation was washed once in 10 ml of medium at 400 X *g* for 10 min and the pellet resuspended in 10 ml of medium, counted using a haemocytometer, and adjusted to 1 X 10^7 cells/ml.

Phagocytosis

The phagocytic ability of collected peritoneal macrophages was measured by uptake of opsonized SRBC. Collected peritoneal cells (approximately 5 X 10^5) were placed in 12 X 75 mm polypropylene Falcon brand tubes (Becton Dickinson and Company, Lincoln Park, NJ) in 900 µl of RPMI-1640 medium with 10% FBS. Opsonized SRBC (1 X 10^6) were added to each tube in 100 µl of medium and the tubes incubated for 2 hr at 37°C. The number of macrophages with ingested SRBC was determined by removing 100 µl of the cell suspension and centrifuging them onto a glass microscope slide at 45 X *g* with a cytocentrifuge. The slides were stained with LeukoStat solutions and 200 macrophages counted to determine the percentage of macrophages with engulfed SRBC.

Experimental design

In all experiments mice were infected on day 0 with 1000 cysts each of *G. muris*. All collections were made in uninfected mice (age matched to infected ones), in mice during the acute phase of infection (10 days after infection), and during the elimination phase of the infection (day 40). Mice receiving the thioglycollate injection were treated three days before collection. Groups of five mice of each sex were used to collect cells at each time period.

B. MEDIATORS

Collection of sera

Sera were collected from mice by cardiac puncture. The mice were killed by cervical dislocation and then bled with a 1 cc syringe fitted with a 23 gauge needle. The blood was placed in 1.5 ml snap cap tubes, left at room temperature for less than 5 min and then placed at 4° C for one to two hours. The samples were then centrifuged at 3000 X *g* for 10 min in a microcentrifuge (MSE Micro-Centaur, Johns Scientific), the serum removed with a Pasteur pipet, and frozen at -20° C until assayed.

TNFα

The levels of TNFα were measured in the serum of mice with a cell killing bioassay using the method of Branch *et al.* (1991). A line of TNF sensitive fibroblasts (L929-8) was kindly supplied by Dr. L. Guilbert, Red Cross, Edmonton and was used in the assay. To perform the assay, 5 X 10^4 L929-8 cells were added to each well of a 96 well plate (Costar cell culture plates) in 50 µl of RPMI-1640 medium (Gibco) with 10% FBS and 2 µg/ml Actinomycin D (Sigma). Actinomycin D is an RNA synthesis inhibitor that prevents the cells from repairing damage caused by the TNF. The cells were then incubated for two hours at 37° C before the samples were added. Fifty µl of samples diluted 1/5 in medium and standards (0.001 to 30 U/ml recombinant TNFα, Genentech, San Francisco, CA) were added to each well of the plate and incubated for 16 hours at 37° C. After incubation 50 µl of the vital dye neutral red (0.05% wt/vol in normal saline) was added and incubated for two hours at 37° C. This dye is selectively taken up by living and healthy cells and not by dead cells. The plate was then washed once with PBS. The addition of 100 µl/well of 0.05 M Na_2PO_4 in 50% (v/v) ethanol released the dye into solution from the cells. After mixing on a rocker for at least 20 min the colour was measured at 570 nm by an automated microplate reader (Bio-Tek Instruments Inc., Highland Park, VT).

Enzyme-linked immunosorbent assay (ELISA) for IFNγ

The wells of 96 well polystyrene plates (Immulon II, Dynatech) were coated with 50 µl of a 1/1000 dilution of the Monoclonal antibody

(MAb) DB-1, diluted in PBS. This MAb is a mouse anti-rat IFNγ which also recognizes murine IFNγ and was obtained from Dr. P. van der Meide, The Primate Center, The Netherlands. The plate was incubated for one hour at room temperature (RT) and then washed. Plates were washed 3 times with washing buffer (PBS with 0.1% Tween-20), using a plate washer (Nunc-Immuno Wash 12), after each step and all reactions were carried out at RT. After this, areas of the wells not coated with antibody were blocked for one hour with 150 µl of blocking buffer (PBS with 1% bovine serum albumin and 0.1% Tween-20) to prevent non-specific binding. One hundred µl of standards (1-50 U/ml recombinant IFNγ, Genentech, San Francisco, CA) and sera diluted 1/5 were added and incubated and, after washing, a rabbit anti-IFNγ poyclonal antibody was added at a 1/500 dilution. This antibody was produced in our laboratory by immunizing rabbits with 50 µg of recombinant murine IFNγ (Genentech) diluted 50:50 with Freunds Complete Adjuvant for the primary immunization. This was followed by several boosts with 50 µg of IFNγ mixed with Freund's Incomplete Adjuvant.

The conjugate used was an HRP goat anti-rabbit Ab at a 1/500 dilution. To develop the colour, 100 µl of 2,2'-azino-di- [3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) (Peroxidase Substrate Kit; Bio-Rad) was added to the wells and incubated for 30 min. The colour reaction was quantified at 405 nm using an automated microplate reader (Bio-Tek Instruments Inc., Highland Park, VT).

Experimental design

In all experiments mice were infected on day 0 with 1000 cysts each of *G. muris*. During the primary infection, sera were collected from 5 infected and 5 uninfected mice (of each sex) on days 10, 20 and 40 after infection. Challenge mice were treated with 1 mg metronidazole each for three consecutive days after the primary infection (days 60-63) to eliminate the infection, rested for 10 days, and then challenged. Challenge serum samples were collected 10 days after challenge.

Challenge infections were done using 5 mice per group. Groups consisted of uninfected mice, mice which had been previously infected and drug cured but not challenged (controls), groups challenged with 1000

cysts and groups challenged with 50 µg of trophozoite extract. The method for the preparation of the trophozoite extract is described in chapter four.

RESULTS

A. PERITONEAL CELLS

Cell numbers

The number of cells recovered from the peritoneal cavity of male and female mice is presented in Figure 5-1. The injection of the thioglycollate resulted in more than a 10 fold increase in the numbers of cells recovered from the peritoneal cavities of the mice. While this treatment did have a significant effect on the cell numbers there was no difference between the males and females (Table 5-1). Ten days after infection there was no overall difference between males and females ($P= 0.96$, three-way ANOVA; Table 5-1) although the infected, untreated females did have significantly more cells than the comparable male group ($P=0.05$, one-way ANOVA; Fig. 5-1). Forty days after infection there was also a significant interactive effect between infection and treatment (Table 5-1). While males had fewer cells than females in the infected, untreated, group, males had higher mean number of cells after treatment indicating that males were responding more than females to the thioglycollate or that females were being more affected by infection than males.

Differential cell counts

The number as a proportion of the total number of cells recovered from the peritoneal cavity was determined for mast cells, eosinophils, neutrophils, lymphocytes and macrophages. Mast cells, eosinophils and neutrophils constituted a small percentage of the total cells recovered from the peritoneum of untreated mice (Fig. 5-2) whereas lymphocytes and macrophages made up approximately 95% of all cells recovered (Fig. 5-3). Treatment of the mice with thioglycollate changed the proportions of the cells present when compared to untreated mice. There were lower percentages of mast cells and lymphocytes and increased percentages of macrophages, eosinophils and neutrophils in the inflammatory cell populations.An examination of each cell type yielded significant

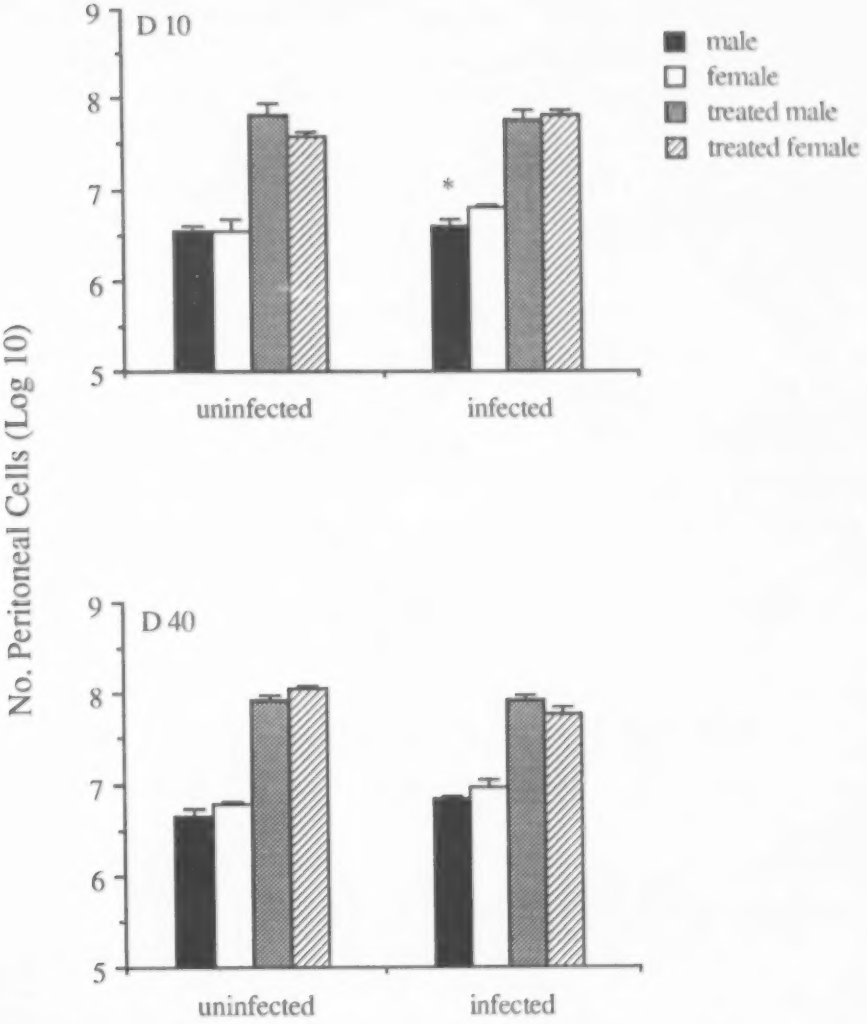

Figure 5-1. Number of cells recovered from the peritoneal cavity of male and female C57BL/6 mice either 10 or 40 days after infection with *Giardia muris*. Treated mice are those which had received an intraperitoneal injection of thioglycollate three days previously. Values are the geometric mean ± SEM of the number of cells recovered for five mice per group except the untreated, uninfected male group on day 40 which has n=4. Asterisks represent significant differences ($P<0.05$, one-way ANOVA) between that group and the corresponding group of the opposite sex.

Table 5-1. Summary statistics for three-way ANOVA analyzing the effect of gender, infection and treatment on the numbers of peritoneal cells in male and female C57BL/6 mice infected for 10 or 40 days with *Giardia muris*.

Ten days after infection

ANOVA Table for log cell #

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	1.149E-4	1.149E-4	.003	.9575
Infection	1	.138	.138	3.456	.0723
Gender * Infection	1	.127	.127	3.193	.0834
Treatment	1	12.863	12.863	323.006	<.0001
Gender * Treatment	1	.098	.098	2.451	.1273
Infection * Treatment	1	.013	.013	.323	.5736
Gender * Infection * Treatment	1	.004	.004	.094	.7611
Residual	32	1.274	.040		

Forty days after infection

ANOVA Table for log cell #

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	.035	.035	2.027	.1646
Infection	1	.005	.005	.314	.5794
Gender * Infection	1	.036	.036	2.104	.1570
Treatment	1	12.121	12.121	707.533	<.0001
Gender * Treatment	1	.042	.042	2.430	.1292
Infection * Treatment	1	.244	.244	14.249	.0007
Gender * Infection * Treatment	1	.050	.050	2.921	.0974
Residual	31	.531	.017		

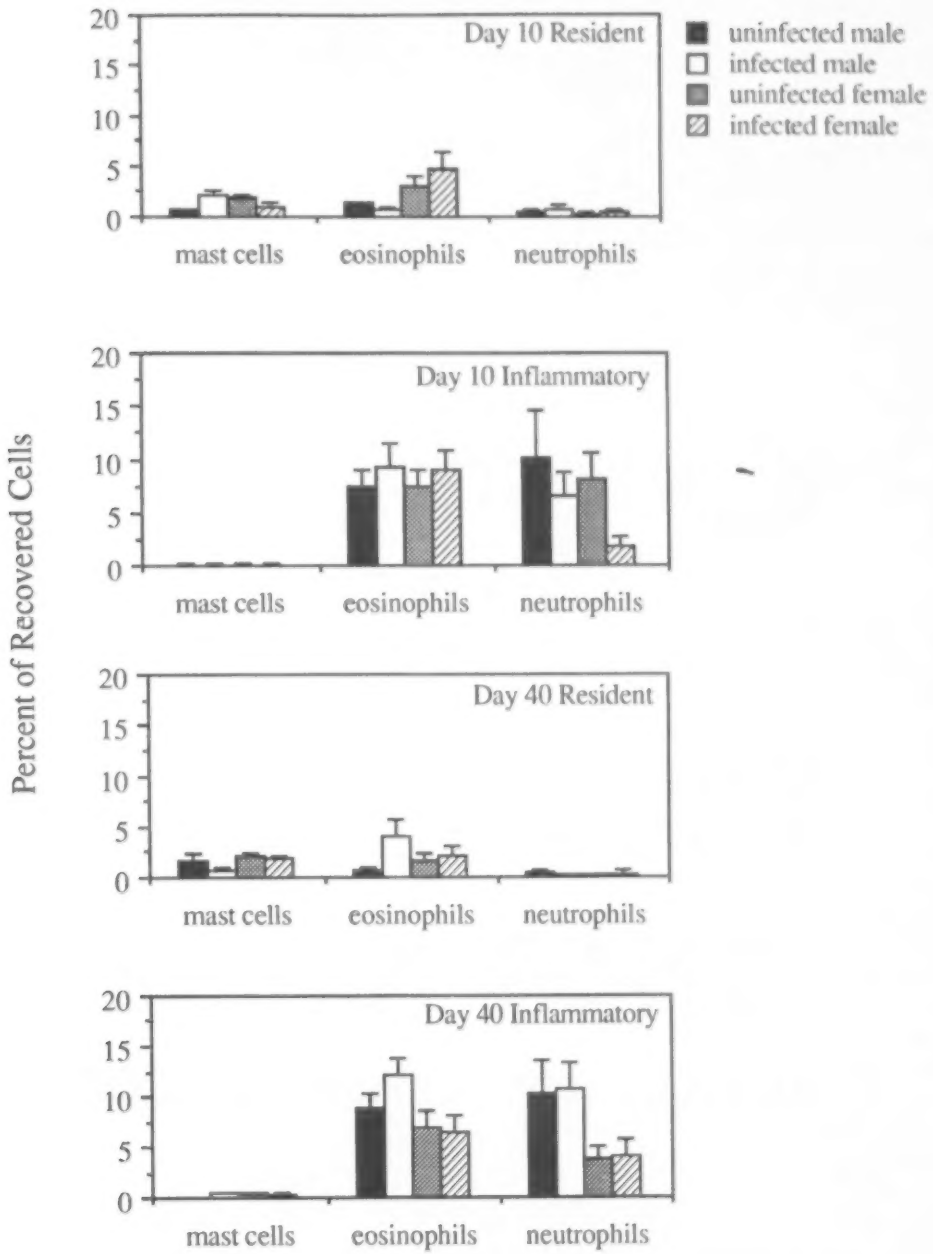

Figure 5-2. Percentages of mast cells, eosiniphils and neutrophils in the peritoneal cavity of male and female C57BL/6 mice during a primary *Giardia muris* infection. Inflammatory cells are those recovered three days after the intraperitoneal injection of an irritant.

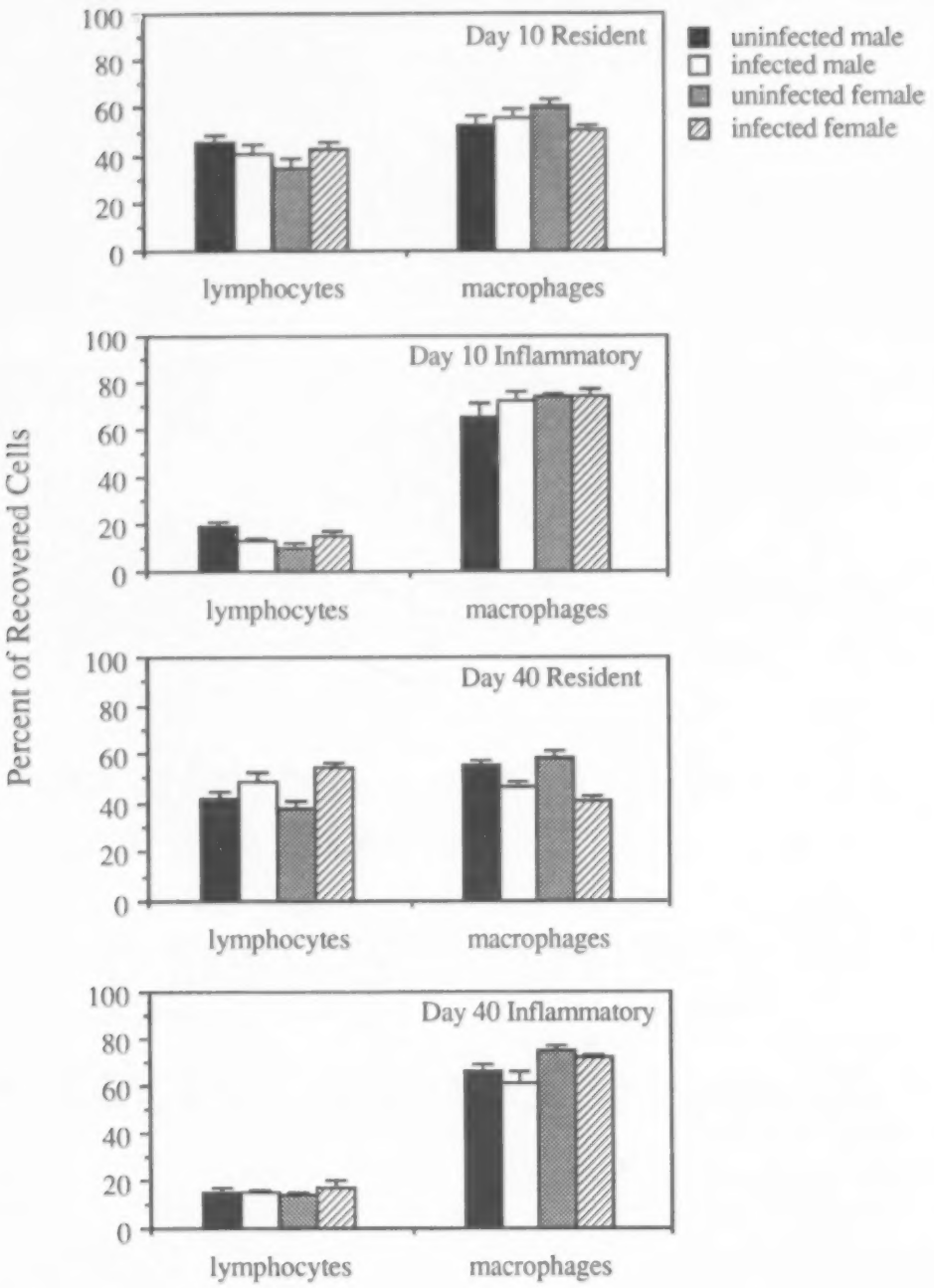

Figure 5-3. Percentages of lymphocytes and macrophages in the peritoneal cavity of male and female C57BL/6 mice during a primary *Giardia muris* infection. Inflammatory cells are those recovered three days after the intraperitoneal injection of an irritant.

differences in some of the cell proportions ($P<0.05$, two-way ANOVA Table 5-2). In the resident populations, there were more eosinophils in the females on day 10. In the inflammatory population, males had more neutrophils and eosinophils than females on day 40 (Table 5-3). On day 40, the resident lymphocytes were significantly influenced by infection with the infected mice having more lymphocytes and fewer macrophages as a percent of the recovered cells.

Phagocytosis of sheep red blood cells (SRBC)

The ability of the macrophages recovered from the peritoneal cavity to phagocytose opsonized SRBC was examined (Figures 5-4 and 5-5). Ten days after infection there were significant differences between males and females and uninfected versus infected mice in their ability to phagocytose the SRBC ($P<0.05$, three-way ANOVA; Table 5-3). Macrophages from females were more phagocytically active than those of males in three of the four groups (Fig. 5-4). The macrophages from thioglycollate treated mice (inflammatory macrophages) were also significantly more active than those from untreated mice (Table 5-3). Forty days after infection, the thioglycollate treatment had a significant effect similar to that seen earlier (Fig. 5-5; Table 5-3). In contrast, the significant effect of gender and infection seen at day 10 was not present on day 40 after infection (Table 5-3).

An inconsistency was observed when the phagocytic ability of inflammatory macrophages of uninfected male and female mice was examined. While on the first examination (Day 10) the uninfected males had significantly lower phagocytic ability than the uninfected females this was reversed on the day 40 examination. The only difference between these mice was their age so I can find no reasonable explanation for this. As well, the phagocytosis of the inflammatory macrophages on day 10 was in the 20 % range while the inflammatory macrophages from day 40 was around 50 %.

Table 5-2. Summary statistics for two-way ANOVA's comparing effect of gender and infection on day 40 inflammatory eosinophils, neutrophils and macrophages from the peritoneal cavity of mice during a primary *Giardia muris* infection. Inflammatory cells are those recovered three days after the intraperitoneal injection of an irritant.

ANOVA Table for eosinophils

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection Status	1	12.013	12.013	.816	.3798
Gender	1	74.113	74.113	5.033	.0394
Infection Status * Gender	1	19.013	19.013	1.291	.2726
Residual	16	235.600	14.725		

ANOVA Table for neutrophils

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection Status	1	.312	.312	.011	.9170
Gender	1	208.013	208.013	7.464	.0148
Infection Status * Gender	1	.012	.012	4.485E-4	.9834
Residual	16	445.900	27.869		

ANOVA Table for macrophages

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection Status	1	94.613	94.613	1.844	.1933
Gender	1	505.013	505.013	9.842	.0064
Infection Status * Gender	1	4.512	4.512	.088	.7706
Residual	16	821.000	51.312		

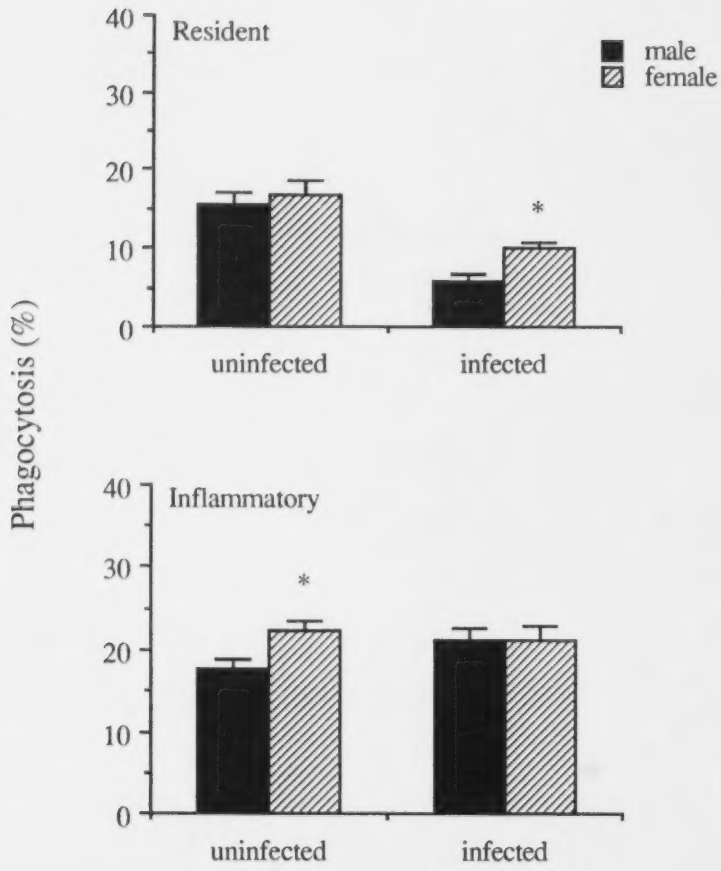

Figure 5-4. Peritoneal macrophage phagocytosis of opsonized sheep red blood cells. Mice had been infected with *Giardia muris* for 10 days. Inflammatory macrophages were those obtained from mice injected intraperitoneally with 1 ml thioglycollate three days previously. Values are the mean ± SEM of 5 mice per group of the percent of examined macrophages with ingested erythrocytes. Asterisks represent significant differences ($P < 0.05$, one-way ANOVA) between that group and the group of the opposite sex.

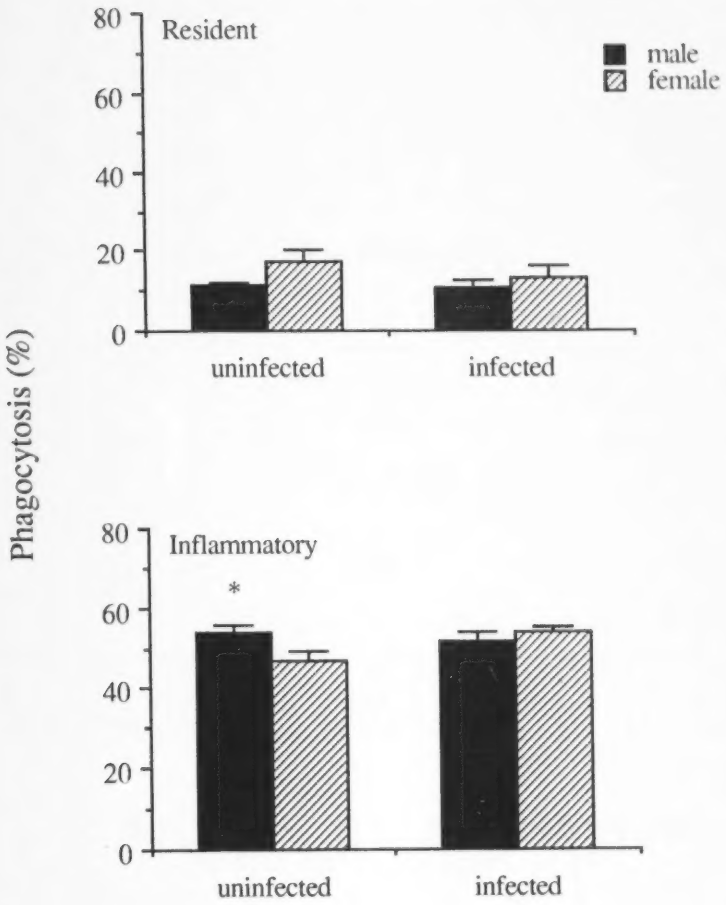

Figure 5-5. Peritoneal macrophage phagocytosis of opsonized sheep red blood cells. Mice had been infected with *Giardia muris* for 40 days. Inflammatory macrophages were those obtained from mice injected intraperitoneally with 1 ml thioglycollate three days previously. Values are the mean ± SEM of 5 mice per group of the percent of examined macrophages with ingested erythrocytes. Asterisks represent significant differences ($P < 0.05$, one-way ANOVA) between that group and the group of the opposite sex.

Table 5-3. Summary statistics for three-way ANOVA analysing the effects of gender, infection and treatment (thioglycollate injection) on peritoneal macrophage phagocytosis of opsonized SRBC 10 and 40 days after infection with *Giardia muris*.

Ten days after infection

ANOVA Table for % phagocytosis

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	63.756	63.756	7.736	.0090
Infection	1	115.600	115.600	14.027	.0007
Gender * Infection	1	1.406	1.406	.171	.6823
Treatment	1	739.600	739.600	89.742	<.0001
Gender * Treatment	1	.506	.506	.061	.8058
Infection * Treatment	1	216.225	216.225	26.236	<.0001
Gender * Infection * Treatment	1	37.056	37.056	4.496	.0418
Residual	32	263.725	8.241		

Forty days after infection

ANOVA Table for % phagocytosis

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	8.550	8.550	.331	.5692
Infection	1	.049	.049	.002	.9657
Gender * Infection	1	25.721	25.721	.997	.3261
Treatment	1	13898.549	13898.549	538.532	<.0001
Gender * Treatment	1	102.688	102.688	3.979	.0552
Infection * Treatment	1	44.652	44.652	1.730	.1984
Gender * Infection * Treatment	1	96.188	96.188	3.727	.0630
Residual	30	774.247	25.808		

MEDIATORS

IFNγ

Mean levels of IFNγ in the serum of the mice never reached detectable levels either during the primary infection or after challenges. The ELISA assay itself gave reliable and predictable results using the recombinant IFNγ standards.

TNFα

The results are not presented as an actual measure of TNFα units in the serum but rather as the measure used to predict this, the percent of cells inhibited. The more TNFα present in the serum the greater the inhibition of the cells. I was not able to get the assay to perform reliably and the values for the controls between trials were quite different between the measurements on day 10 and those on days 20 and 40. As well, the standard curves produced with the standards were not predictable and thus were not reliable. For this reason, the percent of cell inhibition only was used as an indicator of TNFα activity and was not compared between time periods.

On day 10 after infection the infected females had a much higher percent cell inhibition than controls or the males (Fig. 5-6). Significant differences were present between the sexes on days 10 and 40 after infection ($P< 0.05$, two-way ANOVA; Table 5-4). After challenge infections, males had lower mean values than females in all the groups except for the uninfected mice (Fig. 5-6). Males given trophozoite extract had significantly lower values than females ($P=0.028$, one-way ANOVA). Cyst challenge males did not have significantly lower values than females ($P=0.053$).

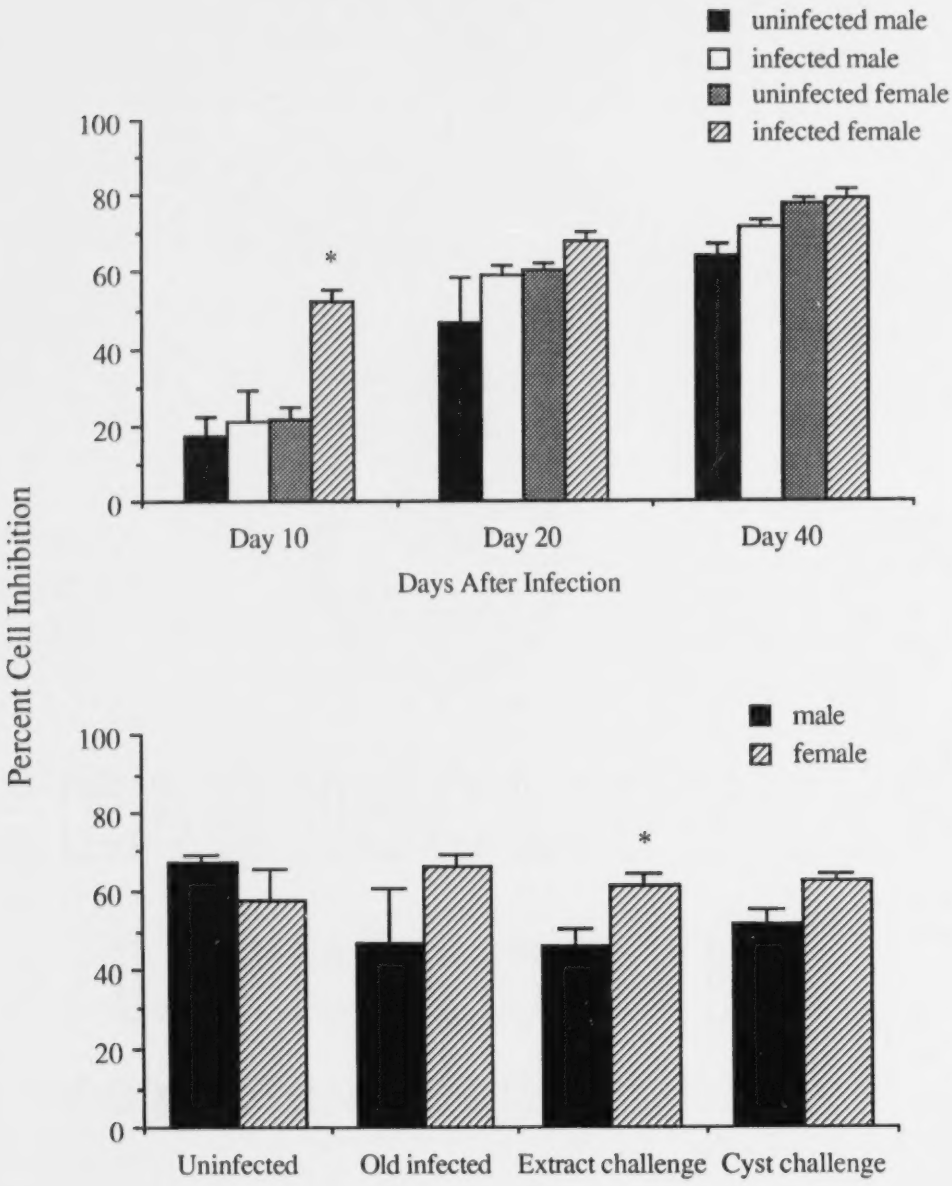

Figure 5-6. Serum levels of tumour necrosis factor in male and female mice as indicated by the percent inhibition of fibroblasts in culture. Values are the mean ± SEM of the percent cell inhibition for both the primary and challenge infection. Asterisks represent significant differences between that group and the corresponding group of the opposite sex.

Table 5-4. Summary statistics for two-way ANOVA analyzing the effect of gender and infection on serum levels of tumour necrosis factor in male and female mice during a primary *Giardia muris* infection.

ANOVA Table for Day 10

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	1540.890	1540.890	11.779	.0034
Infection	1	1426.192	1426.192	10.903	.0045
Gender * Infection	1	859.229	859.229	6.568	.0209
Residual	16	2092.983	130.811		

ANOVA Table for Day 20

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	596.913	596.913	2.928	.1076
Infection	1	463.322	463.322	2.273	.1524
Gender * Infection	1	27.960	27.960	.137	.7163
Residual	15	3057.692	203.846		

ANOVA Table for Day 40

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	516.482	516.482	22.326	.0003
Infection	1	88.804	88.804	3.839	.0703
Gender * Infection	1	45.227	45.227	1.955	.1838
Residual	14	323.870	23.134		

DISCUSSION

In this study, females did not initially have more immune cells in the peritoneal cavity nor did they recruit more cells into the peritoneum after an inflammatory stimulus. The macrophages from females did have significantly higher functional capabilities, as measured by phagocytosis, than did males. The differences in phagocytosis were often small, however, with several hundred thousand macrophages present at an inflammatory focus these differences could be an important influence in parasite elimination.

In a comparative study, Belosevic and Faubert (1986) examined the inflammatory responses of susceptible and resistant mice infected with *Giardia muris*. The susceptible A/J mice recruited significantly fewer cells into their peritoneal cavity after thioglycollate stimulation than the resistant B10.A mice. As well, the macrophages from the susceptible mice were less phagocytically active and less responsive to chemotactic stimuli. Mice of both strains recruited fewer cells when they were infected. The authors concluded from their study that there was a strong relationship between the ability of mice to control *Giardia* infections and their ability to mount *in vivo* and *in vitro* inflammatory responses.

In this study, no major differences were seen between the more resistant female mice and the more susceptible males in their ability to recruit cells into the peritoneal cavity although the macrophages from females were more phagocytically active. An *a priori* assumption based on the study of Belosevic and Faubert (1986) and another comparative study examining inflammation in male and female C57BL/6 mice (Nakanishi *et al.*, 1989) would have led me to expect much greater differences between the males and females in their ability to mount an inflammatory response. C57BL/6 females infected intraperitoneally with the nematode *Brugia pahangi* recruited larger numbers of cells into the peritoneal cavity than did infected males. However, these differences were not apparent until 15 days after infection. It is conceivable that an examination of cell numbers in the males and females at a later time after thioglycollate injection may have shown a noticeable difference in their ability to recruit cells to an inflammatory site.

The apparent lack of IFNγ in the mice in this study has several possible implications. Interferon-gamma has a number of effects associated

with immune function and inflammation. Two subtypes of helper T cells have been identified. The T helper cells associated with release of IFNγ have been designated Th1 cells and also release interleukin-2 and participate in delayed type hypersensitivity responses (Mosmann and Coffman, 1989). IFNγ has an effect on B cell antibody type production and selectively increases the IgG_{2a} subclass while inhibiting other IgG subclasses (Snapper *et al.*, 1988). The levels of IgG_{2a} are not elevated in *G. muris* infections (chapter 4, this thesis) which supports the idea that IFNγ and Th1 cells do not participate in elimination of *Giardia* in mice. Th2 cells release several B cell stimulatory molecules and are probably involved in infections with *G. muris* because of the importance of humoral immunity in this infection (see chapter two). Th2 cells also release another regulatory molecule, interleukin-10, which inhibits Th1 cell proliferation and action and so would therefore inhibit IFNγ production (Mosmann and Moore, 1991). It would be interesting to measure the levels of IL-10 in the mice to test this hypothesis.

Macrophages are major producers of TNFα (Beutler, 1990), a potent cytokine with numerous proinflammatory and immune functions. TNFα increases the functional capabilities of macrophages (Green *et al.*, 1992) and participates in the stimulation of macrophage proliferation (Guilbert *et al.*, 1993). This study found that females produce more TNF than males, especially during infection. This could be an obvious sign of inflammation or macrophage activation in the females which is not appearing in the males. However, because of the technical difficulties described, this apect needs more work before firm conclusions can be made.

Intraperitoneal injections of TNFα induce intestinal disruptions in mice such as villus atrophy and crypt hyperplasia (Garside and Mowat, 1993). This would undoubtedly affect other intestinal functions such as the activities of the brush border disaccharidases. As will be presented in the next chapter, disaccharidase activity after challenges is lower in the females when compared to males. This decrease may possibly be a result of intestinal inflammation brought about by a challenge infection with *G. muris* that induced the release of TNFα from macrophages and caused intestinal disfunction.

Thus, while males and females do not attract more cells to a site of inflammation, the functional capabilities of these cells may be different. The more dramatic differences seen between susceptible and resistant mice by Belosevic and Faubert (1986) were not observed between males and females, indicating that gender differences in inflammatory responses are more subtle than those observed between more genetically distant mice.

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Chapter Six: Disaccharidase activity in the small intestine of male and female C57BL/6 mice after primary and challenge infections with *Giardia muris*.

INTRODUCTION

Abnormalities in small intestinal function and morphology have been identified consistently in human giardiasis and include disaccharidase deficiencies (Jennings *et al*., 1976; Duncombe *et al*., 1978), increased intraepithelial lymphocyte counts (Ferguson *et al*., 1976; Wright and Tomkins, 1977) and villus shortening with crypt hyperplasia (Yardley *et al*., 1964). The small intestinal pathophysiology during *Giardia* sp. infections has been examined in two animal model systems. Decreases in intestinal disaccharidase activities occur in primary *G. muris* infections in mice (Gillon *et al*., 1982; Buret *et al*., 1990; Daniels and Belosevic, 1992) and after challenge infections (Daniels and Belosevic, 1992). Decreases in enzyme activities are also present in both primary and challenge infections with *G. lamblia* in gerbils (*Meriones unguiculatus*) (Belosevic *et al*., 1989). The findings of dramatic decreases in enzyme activities after challenge treatments with crude trophozoite extracts in gerbils (Belosevic *et al*., 1989) and in one of two mouse strains examined (Daniels and Belosevic, 1992) shows that a host response to parasite molecules will cause these decreases in enzyme activity.

The incubation of mouse intestinal mucosal cells *in vitro* with *G. lamblia* trophozoites results in direct damage to the cells and decreased disaccharidase activity (Anand *et al*., 1985). Mice infected with *G. lamblia* show more severe brush border enzyme deficiencies when immunosuppressed (Khanna *et al*., 1988). These studies suggest that the parasite itself can cause the damage to the host. While there are obviously both host and parasite factors involved in disaccharidase deficiencies, the relative contribution of these factors has yet to be elucidated.

Inflammation and pathology in the intestine can result in decreased villus length, crypt hyperplasia, increased cell turnover and shedding of enterocytes, and changes in the biochemistry of the epithelial brush border (Castro, 1990). Disaccharidases are bound to the membrane surface of enterocytes and, therefore, disruptions in villus architecture should result in decreased disaccharidase activity.

Differences in susceptibility to infection are linked to differences in disaccharidase activity in *G. muris* infected mice (Daniels and Belosevic, 1992). We have shown that resistant C57BL/6 females have greater decreases in enzyme activities during the primary infection than the susceptible C3H/HeN females, but the reverse is true after challenge infections. The C3H/HeN mice have dramatic decreases in enzyme activity after challenge with parasite proteins while the C57BL/6 mice had no change in three of four enzymes examined. Male and female C57BL/6 mice could be regarded as susceptible and resistant, respectively, and a similar pattern of differences in enzyme activity might be expected as between the two genetically distinct strains examined previously.

To assess disruptions of intestinal function and indirectly measure immunopathology in the small intestine of male and female mice infected with *G. muris*, the activities of four intestinal disaccharidases were determined. The activities of lactase, sucrase, trehalase and maltase were measured both during primary infections and after challenge infections with either live parasites or an extract of trophozoites.

MATERIALS AND METHODS

Mice

Specific pathogen free (SPF) eight-week old male and female C57BL/6 mice were used in all experiments. The mice were purchased from Taconic Laboratories (Germantown, N.Y.) and maintained under SPF conditions throughout the study.

Parasite

The *Giardia muris* used in the experiments was initially isolated from a Golden hamster by Roberts-Thomson *et al.* (1976). The parasite was maintained in the laboratory by passages of 10,000 cysts/mouse administered by gavage to CD-1/CRBr mice. Passages were done every two weeks.

Isolation of trophozoites from the small intestine

For collection of trophozoites the upper half of the small intestine was removed, slit longitudinally, and cut into 3 cm pieces. The segments of

the small intestine were suspended in phosphate buffered saline (PBS) and agitated with a stirring rod for 30 min at room temperature. The suspension was strained through 20 layers of cheesecloth, centrifuged at 600 X *g* for 10 minutes and the pellet resuspended in RPMI-1640 culture medium (Gibco, Grand Island, NY) containing 20% fetal bovine serum (Gibco). The solution containing trophozoites was incubated at 37° C in glass Petri dishes to allow the parasites to adhere to glass. The supernatant was decanted, PBS added to the dishes, and trophozoites were removed by placing Petri dishes on ice for 10 min. Isolated trophozoites were washed once in PBS by centrifugation at 600 X *g* and stored at -20° C prior to preparation of the soluble extract. Trophozoites were collected from groups of CD-1 mice infected for seven days with *G. muris*. The same procedure was carried out with uninfected CD-1 mice to prepare a control extract solution as the isolated trophozoites still had some bacteria and enterocytes present.

Preparation of soluble trophozoite extract

Trophozoites and the control enterocytes isolated from the small intestine of mice were sonicated for 5 min with a Kontes (Vineland, NJ) Micro-Ultrasonic Cell Disrupter at maximum output. This material was then centrifuged for 5 min at 3000 X *g* and the pellet discarded. Protein concentration of the extract was determined using the Bio-Rad Protein Determination Kit (Bio-Rad , Richmond, Ca.).

Collection of intestinal mucosa

Mice were killed by cervical dislocation and the small intestines removed. Each intestine was divided into four equal sections and the second segment removed and flushed with 40 ml of ice-cold Milli-Q water. The intestinal piece was slit longitudinally, placed on a cold steel slab and the mucosa scraped off with a glass slide, weighed and homogenized in 4 ml of Milli-Q water using a tissue homogenizer. The samples were centrifuged at 600 X g for 10 min, the supernatant removed and frozen at -80° C prior to assay.

Reagents

Substrates were all obtained commercially. Maltose, D(+) trehalose, and ß-lactose were obtained from Sigma Chemical Company (St. Louis,

Mo.). Sucrose and D- glucose were obtained from BDH (BDH Chemicals, Toronto). Other reagents, glucose oxidase, peroxidase, O-dianisidine, Triton X-100 and Tris buffer , were obtained from Sigma. The Tris-glucose oxidase peroxidase (TGO) reagent was prepared by adding 200 mg glucose oxidase, 0.5 ml of 1 mg/ml peroxidase, 1 ml of O-dianisidine solution (100 mg O-dianisidine in ethanol to 10 ml), and 1 ml of detergent solution (20 gm Triton X-100 in 80 gm 95% ethanol) to 100 ml of 0.5 M, pH 7.0 Tris buffer.

Assay for disaccharidases

Intestinal disaccharidase activity was determined using the modified glucose-oxidase peroxidase method of Dahlqvist (1968). Assays were carried out in 96 well microtiter plates in duplicate, and a glucose standard series and reagent blanks were included for each plate. The assay was performed by adding 10 µl of appropriately diluted sample to 10 µl of substrate buffer for the disaccharidase to be measured in duplicate and incubated for one hour in a humidified atmosphere at 37°C. Dilutions of the mucosa were done in Milli-Q ® water and were lactase 1/10 or 1/1, sucrase and trehalase at 1/20 and maltase at 1/70. The disaccharide substrates were made up at a 0.056 M concentration in sodium maleate buffer (0.1 M, pH 6.0). After incubation, 300 µl of Tris-glucose oxidase peroxidase (TGO) reagent was added to each well and incubated for one hour. The addition of the TGO reagent stops the production of glucose by the enzymes and forms a coloured product dependant upon the amount of glucose present. The resulting product was measured at 405 nm in a microplate reader (Titertek Multiscan, Flow Laboratories). Disaccharidase activity is expressed as enzyme units/gram mucosa where 1 enzyme unit hydrolyzes 1 µmole disaccharidase per minute. Depending on the substrate examined then, this measures the production of glucose by the enzyme over a certain time period.

Experimental design

In all experiments, mice were infected on day 0 with 1000 cysts each of *G. muris*. Challenge mice were treated with 1mg metronidazole each for three consecutive days after the primary infection (days 60-63) to eliminate the infection, rested for 10 days, and then challenged.

In the primary infection 5 infected and 5 uninfected mice (of each sex) were collected on days 10, 20 and 40 after infection. Challenge infections were done with 19 females and 14 to 15 males per group. Groups consisted of mice which had been previously infected and drug cured but not challenged (controls), groups challenged with 1000 cysts/mouse and groups challenged with 50 µg each of trophozoite extract. As well, five mice of each sex were challenged with 50 µg of the control extract prepared from uninfected CD-1 mice.

RESULTS

During a primary *G. muris* infection the activities of the four disaccharidases (Fig. 6-1) were significantly influenced by both infection and time ($P < 0.05$, three-way ANOVA; Table 6-1). Only lactase and sucrase had significant differences overall between males and females ($P<0.05$, three-way ANOVA; Table 6-1). However, while females only had decreases on day 10 (one-way ANOVA; Fig. 6-1) males had significantly lower activities of all four enzymes on day 10 and of sucrase, trehalase and maltase (compared to uninfected mice) 20 days after infection. Forty days after infection no changes were observed in the enzyme activities of male or female mice. Thus, a primary infection with *G. muris* resulted in significant decreases of all the enzymes 10 days after infection in both male and female mice but only in males on day 20.

After challenge infections with either cysts of *G. muris* or with trophozoite extract (Fig. 6-2) the activities of sucrase and trehalase were significantly affected by gender ($P<0.05$, three-way ANOVA; Table 6-2) with females having lower enzyme activities after challenge than males ($P<0.05$, one-way ANOVA; Fig. 6-2). The activities of lactase and maltase were not significantly affected by either gender or treatment but showed a significant interactive effect among the two ($P<0.05$, three-way ANOVA; Table 6-2). This indicates that while infection and gender are not being independently affected they are responding differently. For example, females had significantly higher levels of maltase in the old infected group while after challenge males had higher mean levels of maltase activity.

Challenges of the mice with 50 µg of the control extract made of mouse enterocytes had no significant effect ($P>0.05$, one way ANOVA) on disaccharidase activity in male and female mice (Table 6-3).

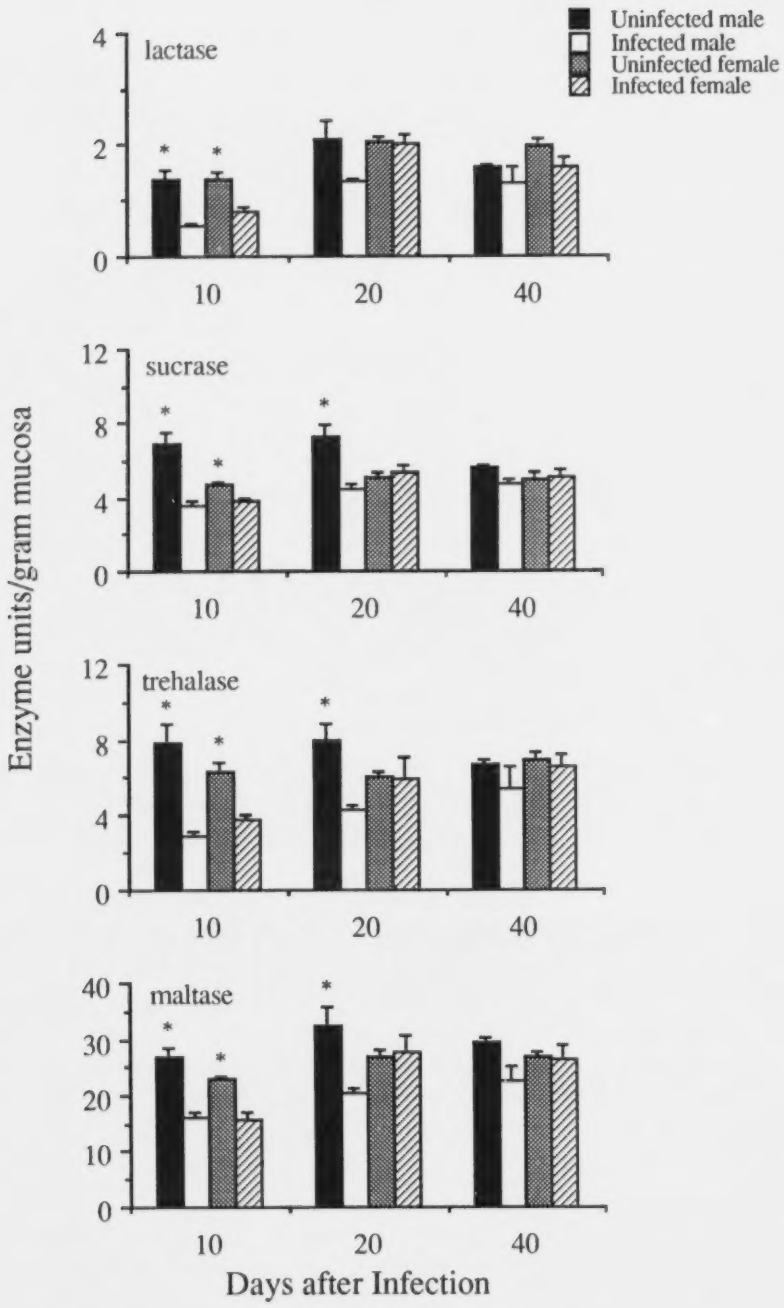

Figure 6-1. Activity of four intestinal disaccharidases in male and female mice during a primary *Giardia muris* infection. Results are expressed as the mean enzyme units/ gram mucosa ± SEM of 4 to 5 mice per group. Asterisks represent significant differences ($P<0.05$, one-way ANOVA) between that group and the other group of the same sex.

Table 6-1. Summary statistics for three-way ANOVA comparing infection, gender and time after infection for four disaccharidases during a primary *Giardia muris* infection.

ANOVA Table for lactase

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	3.367	3.367	28.015	<.0001
Gender	1	.914	.914	7.607	.0083
Infection * Gender	1	.338	.338	2.812	.1003
Time	2	7.186	3.593	29.899	<.0001
Infection * Time	2	.438	.219	1.822	.1732
Gender * Time	2	.140	.070	.580	.5637
Infection * Gender * Time	2	.436	.218	1.813	.1747
Residual	46	5.528	.120		

2 cases were omitted due to missing values.

ANOVA Table for sucrase

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	23.740	23.740	33.313	<.0001
Gender	1	4.610	4.610	6.469	.0144
Infection * Gender	1	16.022	16.022	22.483	<.0001
Time	2	6.015	3.007	4.220	.0208
Infection * Time	2	6.926	3.463	4.860	.0122
Gender * Time	2	1.786	.893	1.253	.2952
Infection * Gender * Time	2	2.763	1.381	1.938	.1555
Residual	46	32.781	.713		

2 cases were omitted due to missing values.

ANOVA Table for trehalase

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	67.092	67.092	32.001	<.0001
Gender	1	.082	.082	.039	.8444
Infection * Gender	1	19.111	19.111	9.115	.0041
Time	2	14.602	7.301	3.482	.0391
Infection * Time	2	21.927	10.963	5.229	.0090
Gender * Time	2	2.511	1.255	.599	.5537
Infection * Gender * Time	2	4.492	2.246	1.071	.3509
Residual	46	96.442	2.097		

2 cases were omitted due to missing values.

ANOVA Table for maltase

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Infection	1	533.264	533.264	31.761	<.0001
Gender	1	.164	.164	.010	.9216
Infection * Gender	1	200.059	200.059	11.916	.0012
Time	2	467.028	233.514	13.908	<.0001
Infection * Time	2	69.366	34.683	2.066	.1383
Gender * Time	2	22.964	11.482	.684	.5097
Infection * Gender * Time	2	51.180	25.590	1.524	.2286
Residual	46	772.324	16.790		

2 cases were omitted due to missing values.

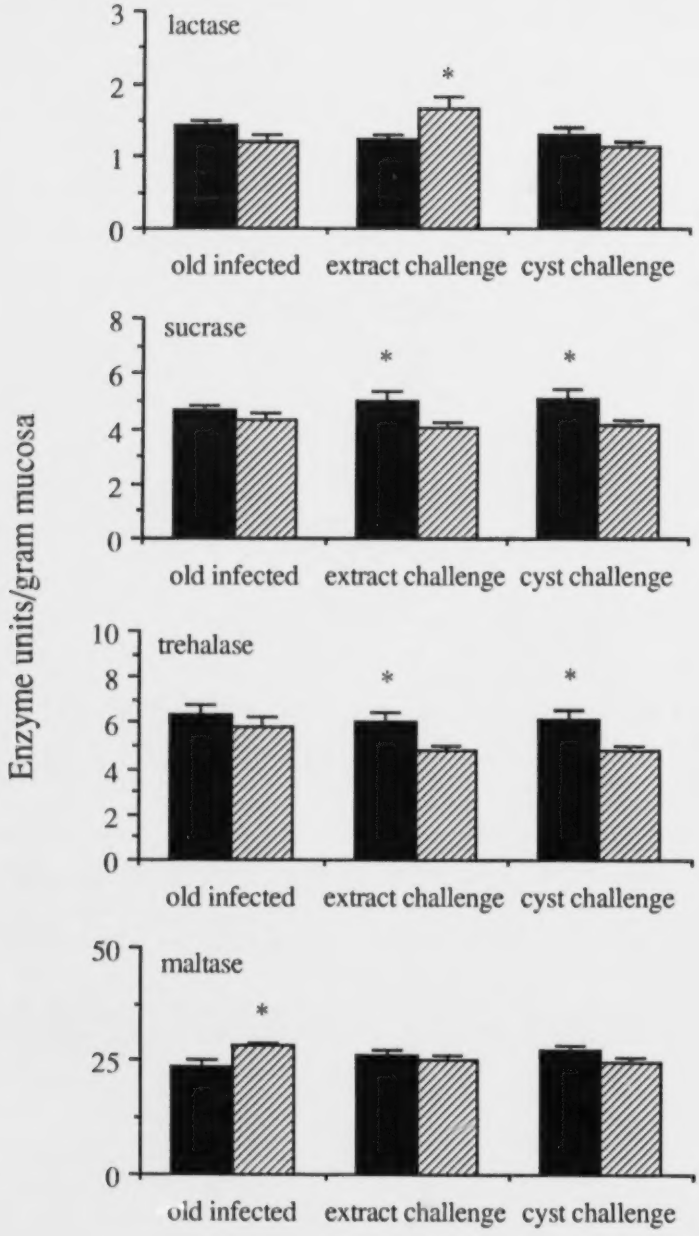

Figure 6-2. Activity of four intestinal disaccharidases in male and female mice after challenge infections with *Giardia muris*. Results are expressed as the mean enzyme units/gram mucosa ± SEM of 14 to 15 male and 19 female mice per group. Solid bars are males and hatched bars are females. Asterisks represent significant differences ($P<0.05$, one-way ANOVA) between that group and the other group of the same sex.

Table 6-2. Summary statistics for two-way ANOVA comparing the effect of gender and treatment on disaccharidase activity after challenge infections with *Giardia muris*.

ANOVA Table for lactase

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	.011	.011	.065	.7988
Treatment	2	.855	.427	2.600	.0796
Gender * Treatment	2	2.142	1.071	6.517	.0022
Residual	95	15.615	.164		

ANOVA Table for sucrase

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	14.002	14.002	13.204	.0005
Treatment	2	.302	.151	.143	.8674
Gender * Treatment	2	2.580	1.290	1.217	.3008
Residual	95	100.742	1.060		

ANOVA Table for trehalase

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	27.643	27.643	14.569	.0002
Treatment	2	10.668	5.334	2.811	.0651
Gender * Treatment	2	3.883	1.941	1.023	.3634
Residual	95	180.253	1.897		

ANOVA Table for maltase

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Gender	1	3.821	3.821	.180	.6727
Treatment	2	.884	.442	.021	.9794
Gender * Treatment	2	221.972	110.986	5.217	.0071
Residual	95	2021.195	21.276		

Table 6-3. Disaccharidase activities in male and female C57BL/6 mice challenged 10 days previously with 50 μg of enterocyte extract. There were 5 mice per group and values are the mean ± SEM expressed as enzyme units/gram mucosa.

	MALE		FEMALE	
	control	challenge	control	challenge
lactase	1.42 ± 0.064	1.24 ± 0.189	1.98 ± 0.208	1.57 ± 0.152
sucrase	4.61 ± 0.228	3.98 ± 0.851	4.01 ± 0.483	5.07 ± 0.294
trehalase	6.38 ± 0.403	5.11 ± 0.525	5.10 ± 0.407	5.71 ± 0.426
maltase	23.80 ± 1.303	24.08 ± 3.008	23.29 ± 2.040	28.50 ± 1.435

DISCUSSION

A primary infection with *G. muris* produced similar decreases in disaccharidase activities in the males and females 10 days after infection, but only in males on day 20. This parallels the information presented in chapter 3 of this thesis on the course of infection. While both sexes have high numbers of trophozoites present on day 10 the females have much lower numbers than males on day 20. This indicates that high numbers of trophozoites are associated with decreases in enzyme activities. While males did have higher numbers of trophozoites than females on day 10 after infection there were obviously enough parasites in both males and females to cause damage to the disaccharidases. My findings are similar to the work of others' who find an association between parasite numbers and enzyme activities. This association is present in *G. muris* (Gillon et al., 1982) and *G. lamblia* (Khanna *et al.*, 1988) infections in mice and in *G. lamblia* infections in gerbils (Belosevic *et al.*, 1989). However, in a study comparing two mouse strains (Daniels and Belosevic, 1992), the susceptible C3H/HeN mice had a higher cyst output in feces than the resistant C57BL/6 mice but their enzyme activities were less affected than those of the C57BL/6 mice. Disaccharidase activities have not been previously reported from male mice.

The mechanisms involved in enzyme deficiencies during infection are unclear. *Giardia* trophozoites have been implicated in direct damage to the intestine during infection both *in vivo* (Belosevic *et al.*, 1989; Buret *et al.*, 1990) and *in vitro* (Anand *et al.*, 1985; Favennec *et al.*, 1991). A diffuse loss of microvillus surface area in the intestine during *G. muris* infections in mice (Buret *et al.*, 1990) and during *G. lamblia* infections in gerbils (Buret *et al.*, 1991) has been noted using electron microscopy. This loss of surface area undoubtedly affects disaccharidase activity. Thus, the higher parasite load in males, during the primary infection, may be responsible for longer and more severe disruptions in intestinal function than in females.

After challenge infections in male and females the trend with the four enzymes' activities is for the female enzyme activities to be more affected than the males. Two of the enzymes were significantly affected by gender and the other two responded differently overall. While the data from other studies suggests a direct effect of the parasite (see above) during primary infection, the findings after challenge infections with crude trophozoite

extracts demonstrates a host response to parasite molecules is involved in decreased disaccharidase activities (Belosevic *et al.*, 1989; Daniels and Belosevic, 1992). In the study with *G. lamblia* in gerbils the decreases in enzyme activity were found as early as day 1 after challenge (Belosevic *et al.*, 1989). Daniels and Belosevic (1992) found decreases in activity of the enzymes as early as 12 hours after challenge of mice with parasite extract. The dramatic decrease after challenge with parasite extract was observed only in the susceptible C3H/HeN mice. The resistant C57BL/6 females had an eventual decline in lactase levels but not until 10 days after challenge (Daniels and Belosevic, 1992). This gradual decrease may have resulted from a slow increase in the host response (inflammation?) after re-exposure to parasite molecules. The findings in this thesis did not confirm this eventual drop in lactase activity in the female mice.

Thus, it appears that parasite numbers are directly associated with disaccharidase deficiency during the primary infection. However, after challenge infections a host response to parasite molecules is evidently involved in disaccharidase deficiency. I observed that male mice are more susceptible to infection than the females but do not have greater decreases in enzyme activities after challenge. The C3H/HeN mice examined by Daniels and Belosevic (1992) were more susceptible to infection than the C57BL/6 females but had greater decreases after challenge with parasite antigens. It follows that the genetic or course of infection differences between genetically distinct strains play a greater role in disaccharidase deficiencies than the gender related differences seen in this study.

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Chapter Seven: General discussion.

In this thesis I set out to answer four main questions: Is the course of a *Giardia muris* infection significantly different in male and female mice? What role do antibodies play in host defense? Is the inflammatory ability of the mice correlated to differential control of the parasite and are the differences in infection related to the pathophysiology of murine giardiasis? I found that differences exist between males and females in their host response to *Giardia muris*. Males are more heavily infected than females, are much poorer antibody producers, and have decreases in intestinal function during a primary infection over a longer time than females. However, the females exhibit greater decreases in enzyme activities after challenge infections. Differences in inflammatory ability between males and females were not marked but the greater phagocytic ability of macrophages from females and their higher levels of tumour necrosis factor suggests a greater inflammatory capacity than males.

SYNOPSIS OF THE FINDINGS

The results of the chapters are interrelated here and are presented on a time basis, with time periods corresponding to the acute, elimination and chronic infection phases.Ten days after infection the parasite load in the intestine and cyst release in the feces were the highest. At this time only antibodies of the IgM class had increased in level and did so equally in males and females. The intestinal environment was altered in both males and females as indicated by the decreased activities of disaccharidases.

Twenty days after infection females had a dramatic decrease in their cyst release. The mean number of trophozoites present in positive males and females had decreased. However, in a number of the females the levels of trophozoites had dropped to undetectable levels at day 20 before increasing again on days 30 and 40 after infection. At this time, on day 20, females had begun showing a strong increase in serum IgG levels while males did not. The subisotypes of IgG had a similar pattern with females having dramatically higher levels of IgG_{2b} and IgG_3 than males. With the decrease in parasite load at this time the disaccharidase activity in the females had returned to normal while the males, with their higher parasite load, still had decreased enzyme function.

Forty days after infection there were no differences in the number of trophozoites in male and female mice although males were still shedding cysts in their feces while females had stopped approximately twenty days earlier. No changes in intestinal disaccharidase activity were observed. The IgM antibody levels were not different from controls, but both males and females had elevated levels of IgG. However, females had a much greater IgG response than males. When the IgG subclasses were measured, the males no longer had the significant increases in IgG_{2b} they had on day 20. Females still had significantly increased levels of IgG_{2b} and IgG_3. The immunoblotting done using sera collected over the course of the infection showed strong responses to several antigens by both males and females. No antigens were ever exclusively recognized by female mice and the differences among individuals were greater than that seen between the sexes.

After challenge infections the males on average shed more cysts in their feces than females, who only occasionally passed a few cysts in their feces two days after challenge. Several males examined though, did not shed cysts in their feces after challenge. The intestinal disaccharidases and antibody responses after challenges suggest that the host responses are different from those occuring during the primary infection. The females had lower disaccharidase activities than males. The females had increases in IgG_{2b} after challenge but not of IgG_3. Indeed, the levels of this subisotype were lower than in males after challenge with parasite proteins.

Obviously then, there is a distinct difference in the specific immune response of males and females. The difference in their inflammatory ability seems unclear and this area requires further work. After the mice were infected, males begin releasing cysts in their feces before the females and at day 10 had many more trophozoites in their intestines than females. The observed differences during the early phase of the infection can not due to a specific immune response but may be due to differences in non-specific host defense mechanisms. These mechanisms could involve inflammation or be due to differences in intestinal physiology between the mice.

BASIS FOR SEX DIFFERENCES IN IMMUNE RESPONSE

The results in this thesis showing dramatic differences between males and females in their antibody responses are hardly suprising. The literature

holds several examples of similar defects in the ability of males to mount effective humoral immune responses (Alexander and Stimson, 1988). Testosterone has been implicated as a major immunomodulator. The outcome of parasitic infections, where males are more susceptible than females, can be modulated by altering sex hormones. For example, in *Brugia pahangi* infected C57BL/6 mice the outcome of infection can be altered by castration of male mice or by administering testosterone to females (Nakanishi *et al.*, 1989). Testosterone influences the immune system in several ways. Administration of testosterone to rodents causes declines in thymus weight and decreases in humoral and cell-mediated immunity. Conversely, castration boosts immunoglobulin levels and thymus weight (Alexander and Stimson, 1988).

In *Plasmodium chabaudi* infected mice, testosterone has several effects on immunity: it increases mortality in the mice, it decreases the number of spleen cells, and it boosts the number of suppressor T cells and decreases the number of B cells in the spleen (Benten *et al.*, 1991). However, once the mice are immune they lose this responsiveness to testosterone and are unaffected by treatment (Wunderlich *et al.*, 1992).

Receptors for testosterone have been identified in the thymus of mice (Alexander and Stimson, 1989). As well, testosterone can be converted by macrophages to its active form dihydrotestosterone. This can then secondarily influence T cells and decrease these cells' release of interleukins (IL) four and five (Daynes *et al.*, 1991). The release of IL-4 and IL-5 has important consequences for humoral immunity as they are potent B cell stimulatory factors (Cox and Liew, 1992).

IMPLICATIONS FOR T CELL CONTROL OF INFECTION

As was discussed in chapter two, a number of experiments have demonstrated the importance of helper T cells in control of *Giardia* infection. The exact nature of this help and the types of cells involved remains uncertain. T helper cells are currently subdivided into two major categories, T helper type 1 (Th1) and T helper type 2 (Th2) cells (Mosmann and Coffman, 1989). These cells were subdivided on the basis of their *in vitro* secretion pattern of lymphokines and on their apparent function. Th1 cells release IFNγ, IL-2 and lymphotoxin and promote delayed type

hypersensitivity reactions. Th2 cells release IL-4, IL-5 and IL-6 and therefore promote B cell activation and growth.

The evidence from this thesis suggests that the Th2 subset of cells participates in the control of *G. muris* infections in mice. Several lines of evidence support the lack of involvement of Th1 cells. IFNγ was not detected in the serum of the mice. IFN-γ stimulates IgG_{2a} responses in mice (Thomson *et al.*, 1993) and this subclass was not elevated in male and female mice. Th1 cells are also involved in delayed type hypersensitivity reactions (DTH) (Mosmann and Coffman, 1989). Examinations of *G. muris* infected mice for DTH responses have proven negative (Ferguson and Munro, 1988). Th1 responses are also generally regarded as being effective against intracellular pathogens, while Th2 cells are often directed against intestinal helminth infections (Cox and Liew, 1992). To date no studies have examined the Th1/Th2 cell types in intestinal protozoan infections although experiments addressing this issue are underway in our laboratory.

FUTURE RESEARCH

In several chapters of this thesis I compared studies using susceptible and resistant strains of mice to *G. muris*, with my own. Male and female mice would be classified as susceptible and resistant, respectively, by the criteria set out by others (Belosevic *et al.*, 1984). However, the differences seen between males and females, in disaccharidase activity and inflammatory responses, have been less than those seen between strains . No comparable data is available for IgG subisotypes but fewer differences were seen in antigen recognition by IgG than that seen between strains (Erlich *et al.*, 1983). The genetic makeup of the host has an obvious importance in resistance of hosts to parasites and is a commonly mentioned and studied factor (Wakelin, 1985). Differences between males and females, however, are found in most systems examined but are often ignored by researchers.This needs to be taken into account in several areas. For example, any attempts to develop vaccines which need to stimulate a humoral immune response need to take into account the gender differences in responsiveness. A vaccine developed without this may leave a large proportion of the target population unprotected and therefore vulnerable.

The work presented in this thesis produced novel information in several areas. None of the work reported in the *Giardia* literature has

examined differences in males and females except in differences in the course of infection. No information was available on the subisotypes of IgG present during infection. The antibody data indicated possible effector functions in humoral and T cell immunity to *Giardia*. Antibody differences between males and females potentially play a major role in the differences in the course of infection observed.

There is now a good body of evidence in the literature regarding control of *G. muris* infections in mice. What has been lacking in the literature are more detailed studies at the host-parasite interface. Reports of the numbers and types of T cells in the intestinal lamina propria, the numbers of Ig cells in Peyers patches and the types of immune cells found in the intestinal lumen have been presented (see chapter 2). However, the exact mechanisms resulting in parasite control remain unknown. Only more detailed studies at the level of the intestine will further expand our knowledge. In addition to the specific immune responses, the role of non-specific host defenses needs to be examined. What role goblet cells, mucus, intestinal physiology, and other factors play in controlling parasite numbers should be examined.

The data from this study on challenge infections parallels the work of others in the course of infection (Underdown *et al.*, 1981; Belosevic and Faubert, 1983) and disaccharidase activities (Belosevic *et al.*, 1989; Daniels and Belosevic, 1992). These results and my data on antibody after challenges point to a fundamental differences in host responses to the parasite in secondary infections. Understanding the mechanisms of host defense after challenge infections would be valuable for control of giardiasis. Humans and other animals would commonly be exposed several times during their lives to *Giardia*. As such, challenge infections are probably more common than primary infections. Therefore, a more detailed examination of host defense and pathophysiology after secondary and multiple exposures to the parasite would be of interest.

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